STRUCTURAL STUDIES IN THE POLYSACCHARIDE GROUP

- by -

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The Constitution and Molecular Structure of Laminarin.

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INTRODUCTION

The utility of seaweeds both as articles of diet and sources of chemicals has long been known. But whereas their use as a foodstuff has gained only restricted popularity - in Japan for example - their industrial usage is widespread and will probably increase when appropriate methods of harvesting and processing have been perfected (1-3).

During recent years the relative importance of the raw materials available from seaweeds has undergone a change. Previously attention has been directed mainly to seaweeds as a source of iodine and potash, but this has given way to an increased interest in their organic constituents. This is due partly to other sources of iodine and potash superseding kelp and partly to the newly recognised utility of the seaweed carbohydrates. One of the latter class of substances, laminarin, is examined in detail in this thesis.

In general, seaweeds may be divided chromatologically into four groups:

Chlorophyceae (green seaweeds)
Phaeophyceae (brown seaweeds)
Rhodo phyceae (red seaweeds)
Cyanophyceae (blue-green seaweeds)

The groups are equally important botanically but, through expediency, only the red and brown seaweeds have been studied to any degree from an industrial or purely chemical aspect.

In addition to inorganic salts, all red and brown seaweeds contain pigments, proteins, other nitrogen-containing compounds, fatty materials and carbohydrate components. Only the last of these, however, have been exploited, mainly because of their colloidal and macromolecular nature. Mannitol, an important sugar alcohol found in most species of seaweed, is an exception.

Seaweed colloids (phycocolloids (4)) may be summarised as follows:

1. Water-soluble polysaccharide ethereal sulphates e.g. agar (R), carragheenin (R), fucoidin (B).
2. Alkali-soluble polyuronides e.g. alginic acid (B).
3. Water-soluble polysaccharides e.g. Floridean starch (R), laminarin (B).

The chief sources of the substances are indicated by the letters R (red seaweeds) and B (brown seaweeds).

The first two groups are probably derived from structural elements in the plant whilst the third probably comprises food reserve materials. In addition, some if not all seaweeds contain algal cellulose (5) which appears to be very similar to the structurally important land-plant cellulose, and a hemicellulosic polysaccharide, xylan (6).

Agar, a complex 1:3-linked galactan which has received much attention from chemists (4-12), is of great commercial value as a gel-forming substance. It is extracted from a wide

variety of red seaweeds and the extraction process is one of the basic industries of Japan (3, 18). Carragheenin is of similar mucilaginous character to agar but differs from it structurally particularly in regard to the high sulphate content of the former. It is obtained by aqueous extraction of Chondrus crispus (Irish Moss) (19, 20, 21), and Gigartina stellata (22, 23). Certain other red seaweeds give well-defined polysaccharides including galactan ethereal sulphates from Dilsea edulis and Irideae edulis (24), Irideae laminarioides (25) and Dumontia incrassata (26). Fucoidin (27, 28), an ethereal sulphate found largely in Fucaceae, forms viscid solutions in water but so far no use has been found for this polysaccharide. Recently (29, 30), it has been found possible to account for all the constituents of the molecule of fucoidin, L-fucose being the only carbohydrate residue, and a tentative structure has been proposed. A polysaccharide similar in properties to fucoidin has been isolated from Macrocystis pyrifera (31).

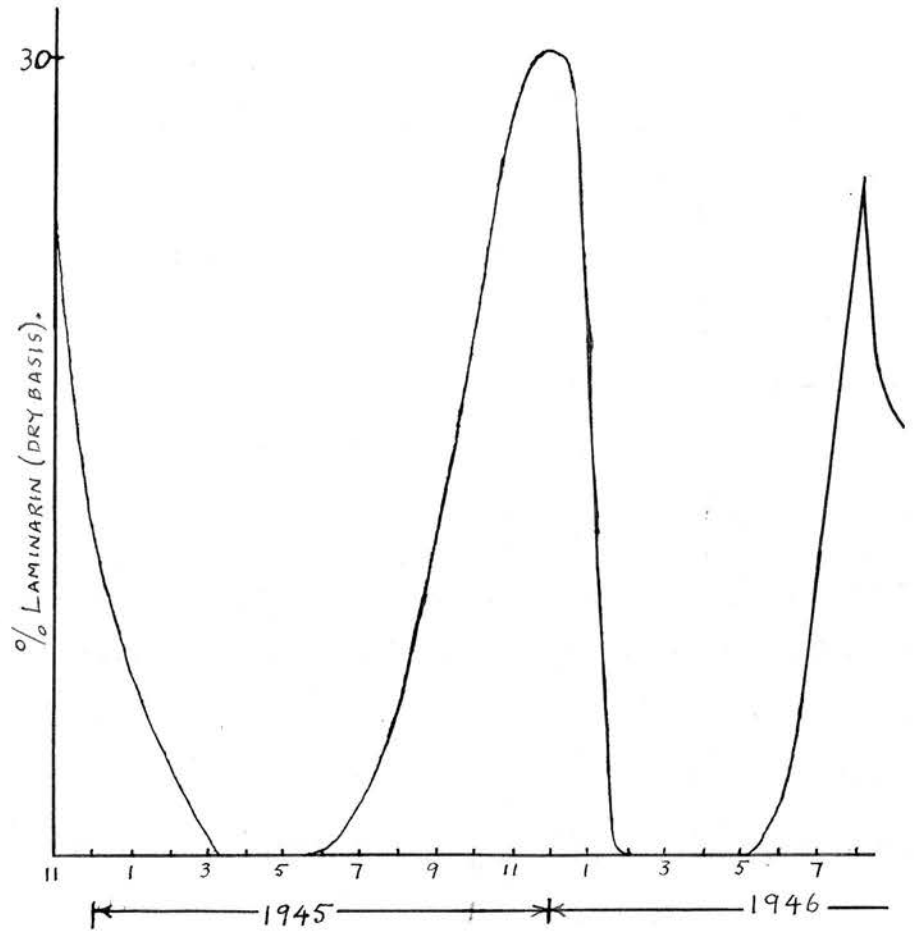
Of the polysaccharides from brown seaweeds, alginic acid (27, 32-41) is, at present, the only industrially important member, finding extensive uses as an emulsifying agent and in fibre manufacture. It is present in all brown seaweeds to a greater or lesser extent and is built mainly, perhaps wholly, of 1:4-linked β-D-mannuronic acid units.

From the chemical standpoint two of the most interesting

seaweed products are Floridean starch and laminarin since together with yeast glucan they are the only glucose polymers having 1:3-links. This type of linkage is widespread in algal chemistry and constitutes a distinct difference between marine- and land-plant polysaccharides in which the linkages are predominantly 1:4. Floridean starch (27, 42) is not a true starch since it does not give the charactistic blue starch-iodine colour and has recently been shown to contain many 1:3-links (43).

Laminarin, the subject of the present investigation, occurs in varying amounts in a number of species of brown seaweed but principally in the laminariales, where it was first observed. Notably, Laminaria digitata, L. flexicaulis, L. saccharina and L. cloustoni are good sources of the polysaccharide. In addition, there is a considerable seasonal variation in the laminarin content of these and allied seaweeds which has received some attention from investigators. Recently Black (44) has made a comprehensive study of the seasonal variation oflaminarin and other components in certain littoral and sub-littoral seaweeds which confirms and extends the former work. Laminarin is a more important component in the sub-littoral (e.g. laminariales) than in the littoral seaweeds (e.g. Fucaceae), where a fairly low and steady seasonal content of 5-9% is observed. In the laminariales, the content may rise to 30% and variation is very much more pronounced (44):

Seasonal variation of laminarin in L. cloustoni



It is generally held that laminarin is a reserve food-material, analgous to starch in the higher plants and glycogen in animals and, like these carbohydrates, it is a D-glucose polymer. It occurs only in the frond of the alga, which in the case of the laminariales builds up a store of the foodstuff during Summer so that the amount reaches a maximum in late Autumn, and utilizes

it during Winter, young leaves having been found to be devoid of it in Spring. Also, the annual seaweed, Saccorhiza bulbosa contains very little or no laminarin (44) as would be expected. It has been suggested that the polysaccharide is a secondary product of photosynthesis from mannitol, which is very probably the primary product, since their seasonal variations coincide quite closely and laminarin does not appear until the mannitol reaches a definite concentration. In this connection Ricard (45) has shown that in L. flexicaulis and L. saccharina, variation in laminarin content follows changes in the duration of sunlight and sea-temperature. It may, however, be utilized also in growth since the laminarin content of the quickly-growing open sea plants is often low and variable, whilst Moss (46) has found that mannitol and laminarin are at a maximum in the growing tips of Fucus vesiculosus, indicating some type of mobilisation.

Since 1885 when Schmiedeberg (47) first described the polysaccharide, obtained by him from Laminaria weeds, few workers have investigated it and until the work of Barry no recorded attempt had been made to elucidate its structure. Krefting and Torup (48) gave an accurate description of a "dextrin-like" carbohydrate which corresponds exactly to laminarin in appearance and properties. This material Krefting

originally extracted from dry L.digitata with cold and then hot water, purifying it from nitrogenous impurities with baryta and lead acetate. Its observed solubility in baryta led Torup to extract it directly from the weed with this reagent, precipitating with acetic acid. Final purification waseffected by repeated spontaneous deposition from water giving a product containing only 0·3% ash and possessing the characteristic granular appearance of laminarin under the microscope. This product was insoluble in cold, soluble in hot water, laevorotary, gave no colour with iodine and was hydrolysed by hydrochloric acid to D-glucose but seemed to be unaffected by the enzymes ptyalin, amylase and diastase.

In the course of the first extensive chemical study of seaweeds ever made, Kylin (48) reinvestigated laminarin, as it became called, and other soluble polysaccharides. It was again extracted with hot water, the solution purified and the laminarin precipitated with alcohol giving a product soluble in cold water but identical in other respects with that of Krefting and Torup. Kylin makes the important claim that laminarin is not a definite polysaccharide but a mixture since by fractionation of aqueous solutions by alcohol he could obtain forms differing in rotatory power, molecular weight, solubility in water and insolubility in alcohol. Fractions having $[\alpha]_D$ -8, -13.5, -22.2 and -32.5° are recorded with molecular weights, determined

cryoscopically, varying inversely from about 3000 to 900. On sulphuric acid hydrolysis 90% conversion to glucose was obtained polarimetrically. An examination of the small free sugar content indicated the presence in certain Fucaceae of a new sugar termed "laminariose" which, however, could not be isolated. It is said to be a disaccharide and is of interest as it may be the laminaribiose of Barry (49), Krefting and Torup having noted that fresh L. digitata contains a laminariase.

Laminarin was again examined by Colin and Ricard (50) but knowledge of the detailed chemical structure was not much advanced. The extraction process, using L. flexicaulis, incorporated both the methods of Kylin and Krefting and Torup, and yielded a product having $[\alpha]_D$ -11.3 or -11.5°; again only 93% conversion to D-glucose was obtained on acid hydrolysis. By cryoscopic determination a molecular size of 6-7 glucose units was proposed giving a molecular weight of 970-1100. Fractions differing slightly in specific rotation were obtained by extracting the product with 70% followed by 60% alcohol, but little importance is attached to this observation. According to these authors, water-soluble complexes of laminarin are formed with barium, calcium, strontium, copper, iron and uranium. It was confirmed that there is little or no attack on the molecule by common enzymes, but snail-juice readily hydrolysed it to glucose.

Laminarin possesses some interesting physico-chemical

properties which have been studied by Gruzewska (51). It has typical colloidal properties, appearing granular under the ultra-microscope, the smallest of these granules exhibiting Brownian movement which is inhibited by alcohol and alkali. Aqueous solutions are unstable and spontaneously deposit macro-particles, apparently through a polymerisation or condensation process for which, it is said, oxygen seems indispensable. The rate of deposition is accelerated by acids and retarded by alkalis, and Barry (52) found that deposition could be prevented indefinitely by glycerol. It is claimed by Mme. Gruzewska that the substance passes slowly through a collodin membrane and that a crystalline modification was obtained as rectangular plates by the addition of alcohol and ether to a 2% solution in 5% sodium hydroxide.

A review of previous observations on laminarin and an examination of the critical points relating to homogeneity of the polysaccharide are contained in the first of a series of important papers by Barry (53). A novel and simple extraction method is described in which Laminaria fronds are steeped in very dilute hydrochloric acid at ordinary temperatures, when white powdery laminarin is deposited on the outside of the plant and as a milkiness in the acid solution. Apparently a soluble modification in the plant is undergoing a change to an insoluble form, identical with that of Krefting and Torup. It is interesting to note that of all the Laminariaceae, L. digitata alone will

not yield laminarin by this treatment and alcohol precipitation must be resorted to. So far, this phenomena has not been explained. The acid-deposited product may be purified by repeated spontaneous deposition from 1% solution in hot water to give a nearly ash-free material. Later (54), Barry separated laminarin from aqueous solution by addition of alcohol which precipitates a water-soluble form similar to that of Kylin. This form gives concentrated aqueous solutions which are water-clear, non-viscid and easily filterable. That these two forms, the water-insoluble and the water-soluble, are chemically similar was demonstrated by preparing their acetates which proved to have identical properties. Previous evidence that glucose is the only sugar obtained on complete hydrolysis was not conclusive, the amounts of this sugar, obtained polarimetrically, never being greater than about 90% when calculated from the theoretical equation:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow n\,C_6H_{12}O_6.$$

By using hydrochloric acid as hydrolysing agent, Barry obtained a 99% yield of glucose polarimetrically, and on destroying the glucose in the hydrolysate with yeast, no optical activity could be observed. Fucose, thought to be the only other sugar likely to be present was excluded on the grounds that no osazone other than glucosazone could be detected on treating the hydrolysate with phenylhydrazine.

It was essential to establish Kylin's claim that laminarin is a mixture of closely-related polysaccharides and with this object in view Barry fractionated the material in a simple fashion. He allowed a 6% aqueous solution to deposit naturally over five days and examined the deposits obtained on successive days for differences in specific optical rotation. The results obtained are shown in Table I:

TABLE I

Successive depositions	$[\alpha]_D^{10}$	Concentration (g./100 ml.)
1st day	-9.85°	2.186
2nd "	-11.26	1.336
3rd "	-10.96	1.600
4th "	-14.12	0.310
5th "	-13.24	0.453

There is no decrease or increase in $[\alpha]_D^{10}$ as might have been expected from this type of fractionation but it was noticed that the specific rotation decreases fairly uniformly with increasing concentration. This was confirmed on a single sample by employing an extended series of concentrations (Table II), and the author suggests that this phenomenon is due to an increase in molecular complexity with increasing concentration, the larger entities being associated with smaller laevorotatory power. The mechanism of this action may be either chemical polymerisation or physical aggregation but the evidence points to the latter since it is found that the apparent molecular weight, as measured by Willstätter and Schudel's method of hypoiodite oxidation (55), does not increase with increasing concentration (Table III):

TABLE II

Concentration (g./100 ml.)	$[\alpha]_D^{10}$
5.436	-6.81
3.624	-8.14
2.718	-8.55
2.536	-9.13
2.450	-10.00
2.304	-10.20
1.812	-10.65
1.596	-10.96
1.433	-11.16
0.994	-11.82
0.717	-12.91
0.453	-13.24
0.359	-13.95
0.239	-14.64

TABLE III

Concentration (g./100 ml.)	Molecular Weight
0.273	5810
0.392	6533
0.406	5042
0.407	5985
0.555	6318
0.707	5253
0.715	5720
0.764	5876
1.148	6042
1.300	5964
1.590	6287
2.026	6535
2.176	6930
2.304	5201
2.408	6320
3.089	5192

The average of these molecular weights, 5884, approximates to a molecule composed of 36 anhydro-glucose units, $(C_6H_{10}O_5)_{36}$ + H_2O = 5850, with a variation of $\pm$ 5 units. Although the results deal satisfactorily with the observations involved in Kylin's claim, no further chemical evidence is offered against a theory that different glucose polymers, possessing similar low specific optical rotations, are present in laminarin.

This facile type of aggregation also probably explains the spontaneous precipitation of laminarin from water. As this aggregation would seem to be purely physical it is difficult to understand the claim of Gruzewska (51) that oxygen is indispensable for the process. It is almost certain that the solubility of the polysaccharide is directly related to the size of its colloidal particles and that the water-soluble forms obtained by alcohol-precipitation owe their solubility to an

extremely fine colloidal condition.

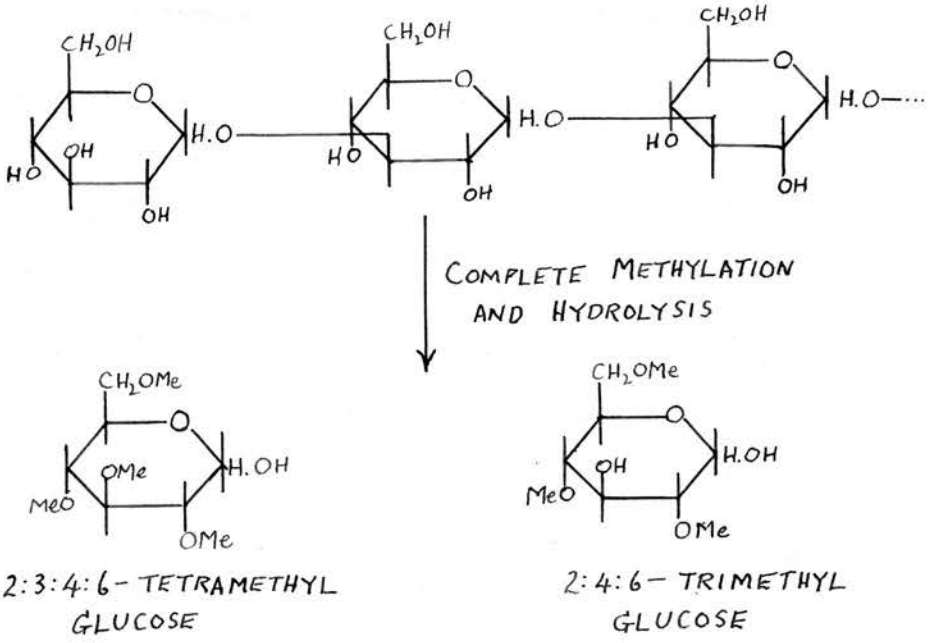
The question arises whether laminarin is present in the living plant in such a condition, perhaps stabilized by some agent which can be inactivated by hydrochloric acid. On the other hand, the widespread occurence of ethereal sulphate groupings in algal polysaccharides has led Barry (53) to propose that laminarin is present in vivo as a complex of this type. The experimental evidence is not conclusive enough to decide with certainty that this is the case, although the proposal explains the observed facts as stated. Laminarin was extracted by a mild method employing 0.25% oxalic acid. The product after purification by alcohol precipitation gave a sulphate-free aqueous solution, but on boiling with 5% hydrochloric acid for 2 hours afforded 0.9% sulphate on a laminarin basis. It was thought that the sulphate may arise from a little fucoidin in the extract, but on hydrolysis no fucose could be detected with phenylhydrazine (54). This low figure is of the order of the sulphate content of agar and, as such, it is significant. Barry, however, appears to consider the product as a nearly completely de-sulphated laminarin ethereal sulphate of the fucoidin or carragheenin type. This is hardly likely but it is clear that the possibility of a laminarin sulphate warrants further investigation.

From his preliminary investigation Barry turned to the

preparation of acetylated and methylated derivatives of laminarin. As stated above the triacetates of both insoluble and soluble laminarin were prepared, the products having similar properties and specific rotations. A theoretical yield of triacetate was smoothly obtained by using a modification of Barnett's method for cellulose, as applied to glycogen by Haworth, Hirst and Webb (56). The product had 44.2% acetyl and $[\alpha]_D$ -52° and on methanolysis gave α- methylglucoside in good yield. A saponified solution of the acetate on acidification deposited the characteristic flaky powder of laminarin and it was presumed therefore that little degradation had occurred during acetylation.

The acetate was used as an intermediate for the preparation of methylated laminarin, presumably to achieve efficiency in the initial stage. Simultaneous de-acetylation and methylation was carried out in acetone at room-temperature using dimethyl sulphate and 45% potassium hydroxide. Seven treatments raised the methoxyl content to 44.7% (theory requires 45.6%), the product having $[\alpha]_D$ -4.4° in chloroform. This methylated laminarin was hydrolysed in the usual manner but a comprehensive search for methylated sugar fragments is not recorded. 2:4:6-trimethyl-D-glucose was identified as a major constituent of the hydrolysate, without the yield being stated. Later (52), mention is made of the isolation of 2:3:4:6-tetramethyl-D-glucose,

representing end-group residues in the polymer, to the extent of 1 in 73 of the total glucose residues. The identification of this trimethyl glucose led to the immediate inference that laminarin is composed in the main of glucose units linked glycosidically through the hydroxyl group on C_3 (I):



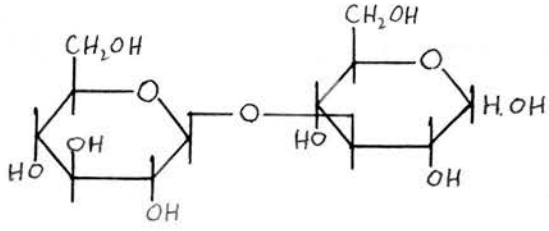
I

The only other recorded glucan which possesses exclusively this unusual type of linkage is that from yeast. Zechmeister and Toth (57) and Hassid, Joslyn and McCready (58) have examined this polysaccharide which furnishes 2:4:6-trimethyl-D-glucose on methylation and hydrolysis. The two polysaccharides have since been closely allied in the literature. Further comparison has been afforded by the isolation of seemingly

identical phenylosazones of disaccharides from the two sources. Zechmeister and Toth hydrolysed yeast glucan with cold concentrated hydrochloric acid and from the hydrolysate were able to separate a phenylosazone of melting point 198°C and specific rotation -75.3° in alcohol, whilst Barry (49), hydrolysing laminarin in various ways, prepared an osazone having values of 195°C and -79.6° respectively. In addition, Barry and Dillon (59) have prepared an osazone from yeast glucan after periodate oxidation which is apparently identical with these compounds. The osazone of the disaccharide, which has been given the name laminaribiose was originally prepared after enzymatic hydrolysis of laminarin.

Previous workers in this field have presented somewhat conflicting views of the action of enzymes on laminarin but all agree that the common enzymes have little or no action. Gruzewska (60) and, later, Colin and Ricard (50) noted that the juice of the snail, Helix pomatia, readily hydrolyses laminarin to glucose and this action was utilised by Barry in searching for a partial hydrolysing agent. Only a small amount of osazone could be obtained in this way and this is attributed to the presence of a laminaribiase which could not be removed. Materials containing laminaribiose itself in an impure form were obtained by the partial hydrolysing action of cold concentrated and hot dilute hydrochloric acid and normal oxalic

acid. Hydrolysis using the first of these reagents could be arrested when a small quantity of the disaccharide was present and after destruction of the glucose by yeast, mixtures of this and oligosaccharides remained. Removal of the oligosaccharides by alcohol precipitation, and subsequent evaporation resulted in a yellow syrup having a low specific rotation, a sweet taste and yielding laminaribiosazone in quantity. From this syrup, on one occasion, colourless rectangular prismatic crystals with a melting-point of 161-2°C were obtained. Most success, however, was achieved with a more controlled reaction using N oxalic acid at boiling-water temperature. Complete hydrolysis required 16 hours (constant rotation) but after 7 hours a maximum quantity of laminaribiose was present as evidenced by the largest yield of the osazone at this stage. An amorphous fraction was obtained from a 7 hour hydrolysate which probably consisted of fairly pure disaccharide since it gave only laminaribiosazone on treatment with phenylhydrazine. It reduced Fehling's but not Barfoed's reagent and could be hydrolysed by emulsin to glucose only, which suggests the glycosidic link.is in the β-configuration. As the previous work indicated that laminarin is a 1:3-polysaccharide, the disaccharide is formulated as 3-[-β-D-glucosido-]-D-glucose (II):



II

The synthesis of what is probably the α-linked isomer has recently been achieved by Gakhokidze (61).

Evidence that the links are β- in the polysaccharide itself is that after 2/3 hydrolysis with oxalic acid the reaction can be completed with emulsin, probably acting on β-links in the oligosaccharides present. In addition, comparison of the rotations of other glucans and their derivatives with laminarin points to the likelihood of β-links in this polysaccharide (Table IV):

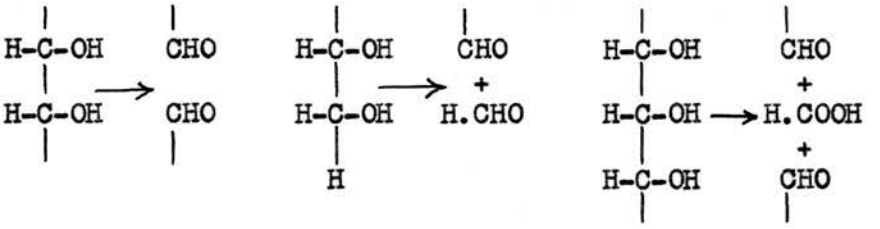
TABLE IV

	Cellulose	Lichenin	Starch	Glycogen	Laminarin
Configuration	β	β	α	α	
$[\alpha]_D$ Polysaccharide	-3.5	+8.3 -2.3	+190	+192	-7 to -16
$[\alpha]_D$ Acetyl derivative	-22	-38.5 -40.3	+170	+163	-52
$[\alpha]_D$ Methyl derivative	-10.0 -4.7	-13.7	+208	+208	-4.4

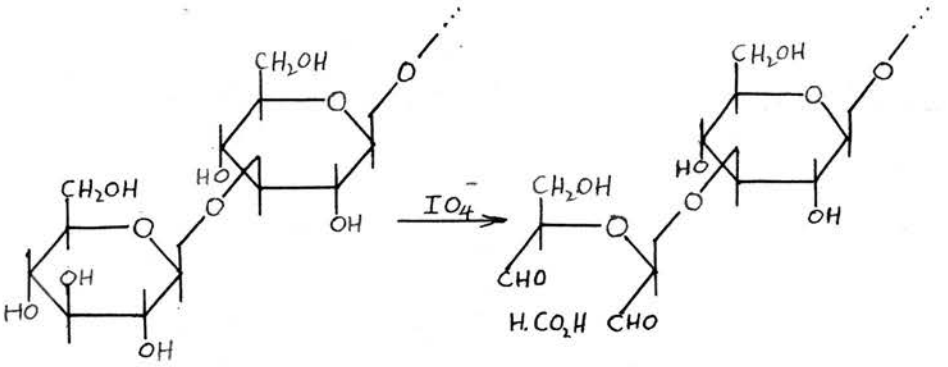
A chain of glucose molecules linked 1:3 in the α-configuration would assume a zig-zag, whilst if in the β-configuration a loop or spiral would result. It is said that the latter, more symmetrical, structure would account for the aggregation and spontaneous deposition of laminarin.

1:3-linked polysaccharides of this kind would have theoretically a very interesting reaction with the periodate ion which reacts in the following well-established manner upon

molecules possessing adjacent $-\overset{|}{\underset{|}{C}}-OH$ groups (62):

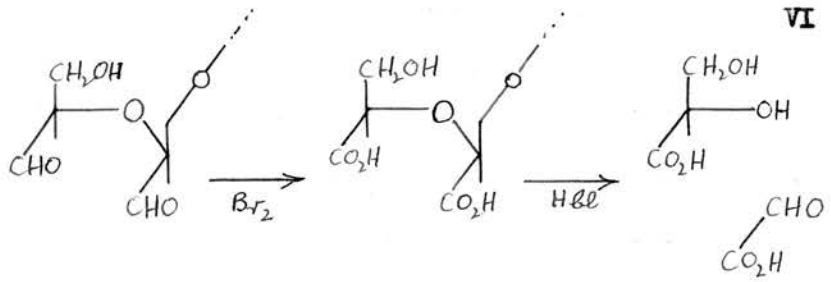


Clearly, none of the intermediate chain-molecules in laminarin will be attacked by periodate since they do not possess the requisite groupings and only terminal end-groups will suffer reaction (III):



III IV

Formic acid is eliminated from every end-group and the dialdehyde IV results. Barry (52) used this particular instance of periodate oxidation as a means of estimating the end-group in laminarin and other 1:3 polysaccharides. Assuming that no other type of linkage occurs so that the number of non-terminal units in the chain remains unchanged on oxidation, the chain-length may be assayed by oxidizing the aldehydo-groups in IV to carboxyl-groups using bromine, and then estimating the acid value by neutralization or by the weight of silver in the silver salt:



IV V VI VII

The "di-carboxylated" material (V) so obtained, termed laminarinic acid, was hydrolysed with hydrochloric acid and the liberation of a small quantity of glyoxalic acid (VII) was demonstrated. Also, oxalic could be detected in the hydrolysate when this was oxidized further with bromine.

After bromine oxidation, the laminarinic acid is easily isolated since, like laminarin, it flakes out of solution and, in fact, cannot be distinguished in its ordinary properties from the parent polysaccharide. The dried product gave the following results (Table V):

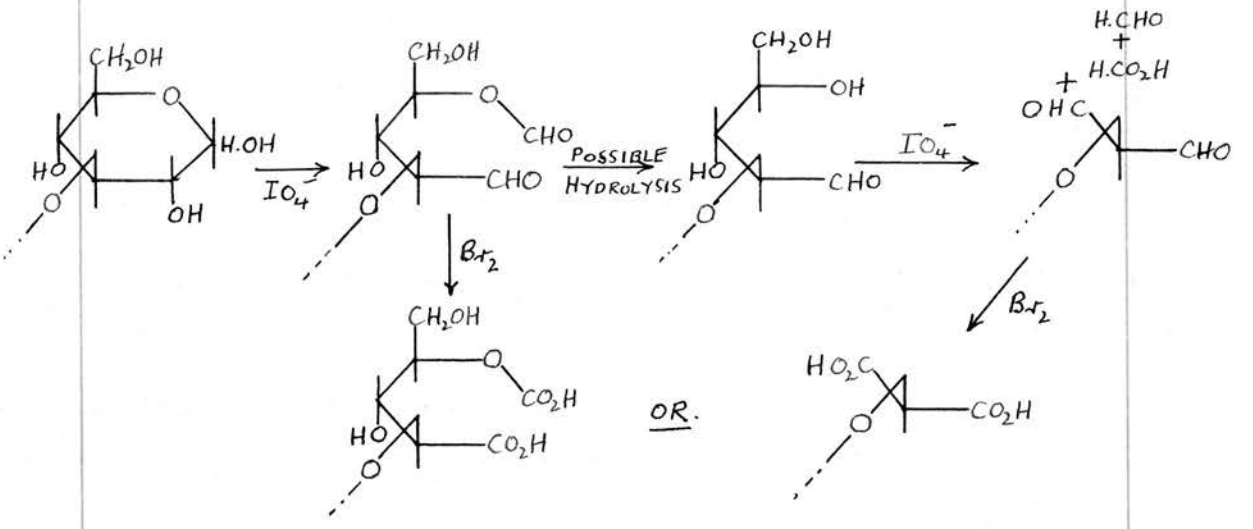
TABLE V

Neutralization value (weight of laminarinic acid to neutralize 2L. of N sodium hydroxide)	Molecular Weight (weight of silver laminariate which contains 216g. silver)
2553	2580
2474	2421
2778	2739
2642	2730
2556	2578
2554	2637
2663	2604
2424	2613
2666	

Mean Molecular Weights: 2553 and 2606

A Molecular Weight of 2550-2600 corresponds to a chain-length of about 16 anhydro-glucose units in the parent laminarin.

The action of bromine also oxidizes the aldehydic end-groups to carboxyl groups and allowance is made for this excess carboxyl content by oxidizing laminarin itself under parallel conditions, but this allowance is not strictly valid since the aldehydic, reducing end-groups are already modified by the periodate:



Further dicarboxylated end-groups are thus probably obtained which would not be the case if bromine oxidation alone were effected, and since laminarin has an appreciable reducing power, the effect of these modified groups would be by no means negligible. Moreover, the work of Halsall, Hirst and Jones (63) has shown that the conditions of periodate oxidation must be carefully controlled in order that over-oxidation may be prevented or taken into account. With this in mind, it is probably necessary to apply more rigorous experimentation to the method.

The position so far, then, is that laminarin is probably a single distinct polysaccharide composed wholely of glucose residues joined for the main part by 1:3-β-glycosidic links and that the chain-length is fairly small.

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DISCUSSION

The laminarin used in the present investigation was kindly given by the Scottish Seaweed Research Association. It was extracted from fresh L. cloustoni fronds collected at Oban in October/November, 1947.

The seaweed was chopped into small pieces, covered with dilute hydrochloric acid (30 mls. concentrated to 1000 ml. water) and allowed to stand for 2 days. The mixture containing the finely suspended laminarin was agitated, filtered and the weed extracted a second time with dilute hydrochloric acid. The combined extracts were allowed to stand for several days when the laminarin settled out. The clear supernatant liquor was siphoned off and the laminarin dissolved in hot water at 80°C and filtered from the weed residue. The laminarin, which separated as a fine precipitate, was filtered off, redeposited from hot water, washed with alcohol and ether and then dried in a vacuum desiccator. It contained 10% moisture, gave 0.4% sulphated ash and had $[\alpha]_D^{15}$ -14.4° (c, 0.9 in water).

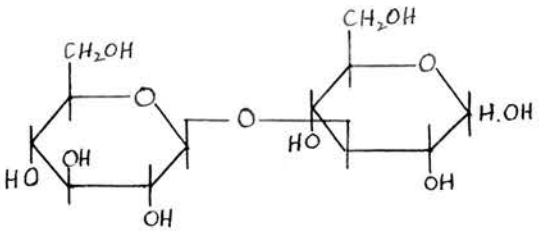
The original sample as received contained some chloride but no sulphate ions, and was purified by spontaneous deposition from 0.6% solution in hot water (50°C). After four depositions a product (Fraction A) was obtained having only 0.2% ash and giving negative tests for both sulphate and chloride ions. As the recovery in this purification was only 57%, the combined depositing solutions were evaporated to small bulk under reduced

pressure and again allowed to stand. No deposition occured, however, and so the remaining dissolved laminarin was precipitated with alcohol giving a water-soluble product (Fraction B). Fraction A: 24% moisture, 0.2% ash and $[\alpha]_D^{15}$ -14.8°(c,0.76 in water) and Fraction B: 15.6% moisture, 0.2% ash and $[\alpha]_D^{16}$ -12.9° (c,0.85 in water). These rotations agree fairly well with those previously recorded, but their small value and variation with concentration (1) makes them unsuitable for exact comparison.

Hydrolysis of deposited laminarin with hydrochloric acid at 95°C was completed (constant rotation) in 3 hours when 96% glucose was found polarimetrically and between 93 and 95% reducing hexose by hypoiodite oxidation. The neutralised hydrolysate when examined by the method of paper chromatography (2) showed the presence only of glucose, thus verifying conclusively the previous view that laminarin is composed wholely of D-glucose residues. In addition to decomposition the low analytical figures may be due to the presence of small amounts of non-reducing substances or to incomplete drying of the polysaccharide. The former possibility has not been investigated but is not considered likely since no derivatives other than those of glucose have been encountered in subsequent work.

By partial hydrolysis of laminarin, Barry obtained the structurally important disaccharide laminaribiose in a mixture of glucose and glucose polymers (see Introduction) but was unable to separate it in a pure form. The pure material has now been

obtained from such a mixture by the elegant method of partition chromatography as applied to the bulk separation of sugars (3). Following Barry's work, N oxalic acid was chosen as the hydrolytic reagent and the course of hydrolysis followed by periodically examining portions of the neutralised hydrolysate on the paper chromatogram. The disaccharide developed as a distinct spot having an R_F value about 0.4 that of glucose and, particularly when using fast-running chromatogram solvents, the presence of a trisaccharide was evident in small amounts. The concentration of laminaribiose, as judged by the intensity of the spots on the chromatogram, was at a maximum after about 7 hours hydrolysis. Using this optimum time a mixture of glucose, laminaribiose and oligosaccharides was obtained which was separated with partially saturated n-butanol on a column of cellulose. Attempts to remove the glucose by yeast fermentation were abandoned since they appeared to lead to much destruction of the disaccharide. Laminaribiose, representing about 15% of the mixture, was obtained as a deliquescent glass from this separation. The glass, which could not be readily crystallised analysed as a dihydrate and melted slowly at 160-163°, losing water at about 80°. The optical rotation was small, +19.0° (c,2.7 in water), indicating that the glucosidic linkage is in the β-configuration:



I

Gakhokidze (4) has synthesised what appears to be 3-α-D-glucosido-D-glucose having $[\alpha]_D^{18}$ +84.8° in water, and the feasible synthesis of the β-form for comparison would confirm the existence of β-links in laminarin. The osazone of laminaribiose was prepared in the usual manner and had m.p. 200-202°, $[\alpha]_D^{14}$ -71.5° (c,0.5 in ethanol), and analysed as the dihydrate of a disaccharide osazone. Barry (5) records m.p. 195° and $[\alpha]_D^{10}$ -79.6° in ethanol for laminaribiosazone and the disaccharide osazone of Zechmeister and Toth (6), which is probably identical with laminaribiosazone, had m.p. 198° and $[\alpha]_D^{20}$ -75.3° in ethanol. Material which is largely a trisaccharide has also been obtained by prolonged elution of the cellulose chromatogram.

Acetolysis has often been used with profit in the elucidation of polysaccharide structures and this reaction was applied to laminarin with a view to obtaining an octaacetyl laminaribiose. The usual method of preparing octaacetyl cellobiose from cellulose when tried with laminarin yielded a product having an average chain-length of about 4 units. With more vigorous conditions small amounts of non-crystalline material were obtained

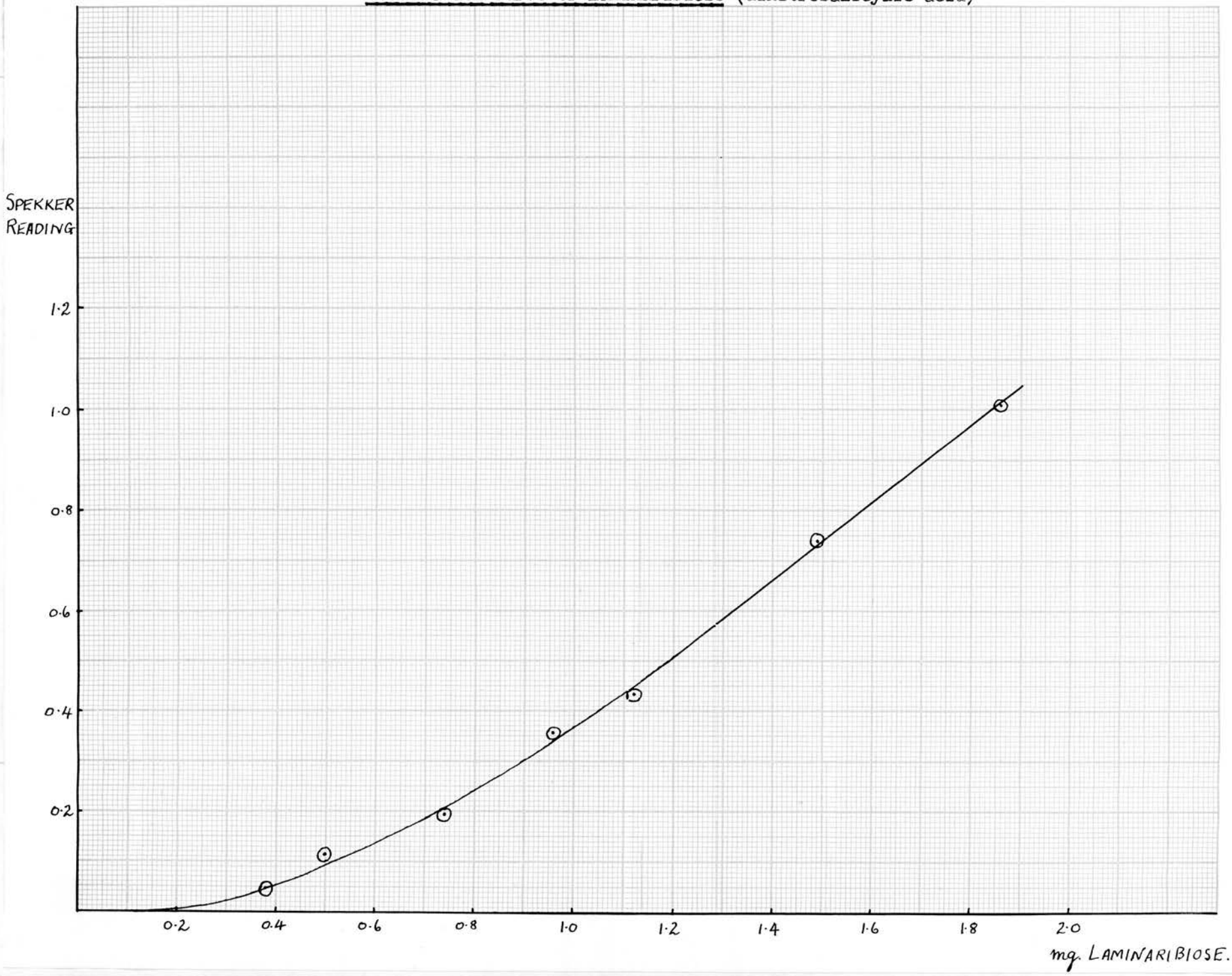
having indefinite melting-points between 40 and 100°. The presence of acetylated disaccharides in these materials was established by paper chromatography following saponification, but in view of the success of the above partial hydrolysis the method has not been pursued.

Laminarin has an appreciable reducing power, and Fehling's solution is readily reduced after about 10 seconds boiling. Barry states (7) that he could find no stoicheiometrical relation for the reducing power when determined by the hypoiodite method, but in view of the importance of this property it was re-investigated. The method of Willstätter and Schudel (8), employing strong alkali, gave reproducible results when the reaction was carried out in homogeneous solution by first rendering the laminarin soluble and working before deposition had started. Contrary to Barry's observation it was found that, within the experimental error, the degree of oxidation was independent of variation in concentration of laminarin and duration of oxidation. Thus in six experiments, the consumption of iodine per 1g. of laminarin was 3.13, 3.06, 3.20, 3.06, 3.13 and 3.10 ml. 0.1N iodine, respectively. The mean of these values, 3.11 $\pm$ 0.05 ml., corresponds to the presence in the molecule of 1 reducing end-group per 40 $\pm$ 1 non-reducing residues. Oxidation in buffered alkali with similar reaction times gave higher figures of about 47 residues, but on

prolonged oxidation (18 hours) a comparable result of 41 residues was obtained. The reducing end-groups in degraded celluloses have been estimated by oxidation with alkaline copper sulphate and determination of the methylene-blue binding power of the acid product (9). Similarly, a definite oxidation of the free reducing group in laminarin was observed when it was treated with bromine, and the resulting oxidised product could be readily and directly titrated. The neutralisation equivalent of oxidised laminarin so determined corresponded to 1 carboxyl group per 48 residues, which agrees fairly well with the above reducing values. The reducing power of laminarin is clearly quite large compared with that of most polysaccharides and, as such, ought to respond well to the recent method of molecular weight determination developed by Meyer, Noelting and Bernfeld (10). This method depends upon the reduction in hot (65°) alkaline solution of 3:5-dinitrosalicylic acid to a highly coloured compound, probably 5-nitro-3-aminosalicylic acid. The intensity of colour produced by known concentrations of reducing substance is measured photometrically and an intensity/concentration calibration curve drawn up using a model substance of known polymeric size - in the case of starches, maltose was employed. Direct comparison of the intensity of a known weight of polysaccharide with the curve, enables the molecular size to be immediately calculated. With laminarin the appropriate model substance is laminaribiose since it reproduces the 1:3-β-links of the polysaccharide, and the accompanying graph I (p. 32) shows a calibration curve for this disaccharide. Using this curve ff

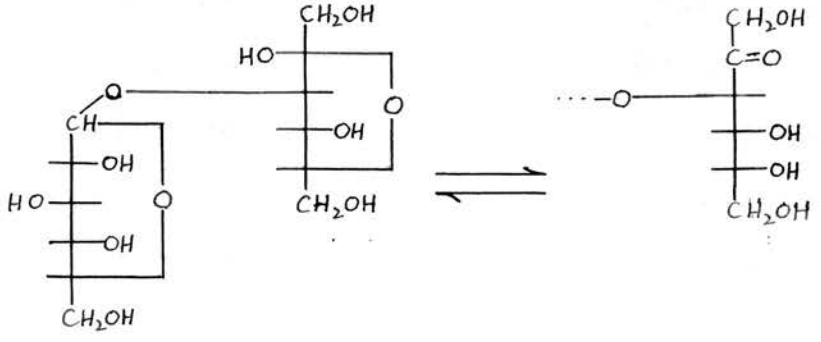
GRAPH I

Calibration Curve of Laminaribiose (dinitrosalicylic acid)



a molecular size (degree of polymerisation) of 13 to 14 glucose residues was obtained. Apart from the fact that the reaction between dinitrosalicylic acid and carbohydrates has not been fully defined, an explanation of these lower values may be an alkali-sensitivity of laminarin. Ross (11) has shown that the reducing power of laminarin as measured by hypoiodite oxidation, rises considerably after heating with strong alkali; in one experiment an apparent chain-length of 8 was observed after pre-heating at 65° in 2N sodium hydroxide for 30 minutes. Such an apparently remarkable sensitivity to hot alkali is therefore likely to invalidate the results with dinitrosalicylic acid.

The normal glycosidic link both in glycosides and polysaccharide is generally quite stable to alkali and the behaviour of laminarin, whose only peculiarities are its relatively small molecular weight and 1:3-linkages, is inexplicable at present. The analogy to the to the disaccharide turanose is easily drawn but is not really valid. Turanose, 3-[-α-D-glucosido-]-D-fructose (II) is readily hydrolysed by alkali (12):



II

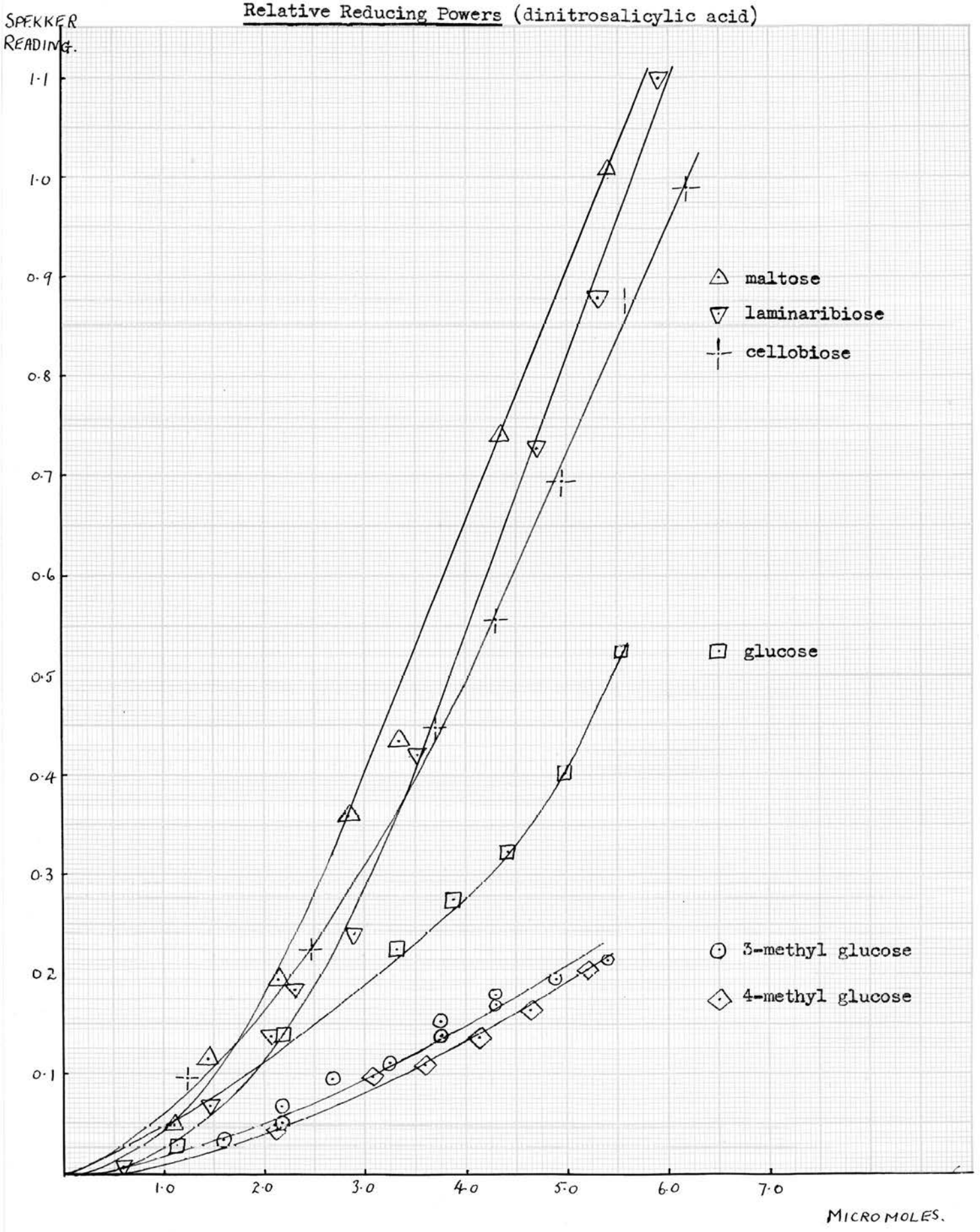
However, it is not the 1:3-linkage as such but probably the proximity of the carbonyl group which renders the glycosidic link susceptible to alkaline hydrolysis. Periodate oxidised celluloses, which contain the system: -O-CH(HC=O)-O-glycosyl, are similarly degraded by alkali (13, 14). Laminarin contains no carbonyl groups adjacent to the linkage, except, perhaps, an incipient one formed by epimerisation of the reducing end-group - an event which it is difficult to imagine leading to extensive degradation. No such degradation is apparent with laminaribiose as is seen from an examination of the graph II (p.35) of the relative reducing powers of this disaccharide and others as measured by the dinitrosalicylic acid method. Laminaribiose is slightly more reducing but not significantly so.

The question of the stability of laminarin in cold, as opposed to hot, strong alkaline solution must be posed since the structurally important methyl derivative (to be described) has been prepared in this medium. The properties of the methyl derivative suggest, however, that little or no degradation has occured in its preparation. The molar specific viscosity of methyl laminarin is greater than that of the acetyl derivative which has only a slightly greater reducing power than laminarin itself.

Graph II also shows the molar-reducing powers of the disaccharides relative to glucose and some glucose derivatives.

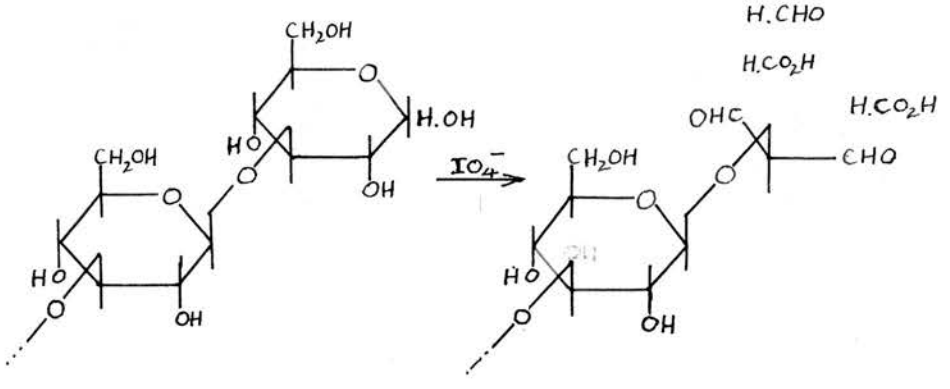
GRAPH II

Relative Reducing Powers (dinitrosalicylic acid)



It appears that the disaccharides have a greater and the methyl derivatives a smaller relative reducing power than glucose. In addition, the introduction of more methyl groups into the glucose molecule has a progressive diminishing effect on the reducing power, 2:4:6-trimethylglucose giving hardly any reduction in similar molar concentrations. The position of the substituent appears to have little effect: laminaribiose is slightly more reducing than the other disaccharides and 3- and 4-methyl glucose are almost equally reducing though it must be admitted that in this case insufficient 4-methyl glucose was available for an adequate comparison. A simple explanation of these differences is not possible in terms of electronic shifts and it is likely that a complex break-down of the dienol forms of the carbohydrates is involved in the strong alkaline conditions.

Mention must be made at this stage of a novel determination of reducing end-groups in laminarin which is independent of reducing power. When a 1:3-linked polysaccharide is oxidised by periodate the only primary hydroxyl group in the molecule to be affected is that of the reducing end-group:



This type of oxidation, which will be fully discussed later, yielded formaldehyde from the primary hydroxyl group equivalent to the presence of one reducing group in about 27 residues.

Laminarin has been acetylated by three methods and the products compared. Barry (15) employed a modification of Barnett's method for cellulose as applied to glycogen by Haworth, Hirst and Webb (16) and this method was repeated - giving a 68% yield of a product (I) having 43.9% acetyl and $[\alpha]_D^{16}$-58.8° (c,1.1 in chloroform). Milder conditions were then tried using acetic anhydride in pyridine. Attempts to disperse the polysaccharide in pyridine by the procedure of Pacsu and Mullen (17) failed because sufficient water could not be distilled off azeotropically without precipitation of the laminarin and on acetylation of the aqueous pyridine solution a product (II) having only 29.3% acetyl, $[\alpha]_D^{18}$+3.9°, was obtained. A satisfactory preparation was achieved by dispersing alcohol-precipitated laminarin directly in pyridine and acetylating as usual, when a product (III), 44.0% acetyl, $[\alpha]_D^{15}$-59.8° (c,0.96 in chloroform) resulted.

Acetyl products I and III were compared by measurement of their reducing powers using Bergmann and Machemer's method (18), and of their viscosities in m-cresol. The former method gave figures of 1 reducing group per 22 and 35 residues, respectively, in the saponified acetates. Although the method is not very

accurate, the order of the reducing powers indicates that the chain-length of acetyl product I has been reduced with respect to laminarin itself, whilst that of acetyl product III is about the same. The viscosities of the acetyl and methyl derivatives (to be described) reflect the same degradation:

	Acetyl I	Acetyl III	Methyl I	Methyl III (average)
η_{sp} in m-cresol (20°, 2%)	0.152	0.266	0.248	0.305
$\frac{\eta_{sp}}{M'}$	529.10^{-4}	925.10^{-4}	1215.10^{-4}	1500.10^{-4}

η_{sp} = specific viscosity

M' = molecular weight of a unit residue

The molar specific viscosity of acetyl laminarin I is less than that of III showing it to be more degraded, and both are less than either of the methyl derivatives which would be expected to be least degraded. The degraded nature of product I is not surprising in view of Higginbotham and Richardson's observation (19) that starch acetates prepared with the aid of Barnett's reagents are degraded.

Prolonged treatment of laminarin with benzoyl chloride in pyridine resulted in a product analysing by acyl determination and combustion, as a monobenzoyl derivative. This material had $[\alpha]_D^{14}$ -54.9° (c,1.03 in chloroform), was difficultly saponifiable and was insoluble in water, ether and alcohol but readily soluble in acetone and chloroform from which it could be precipitated with petrol.

The major part of this thesis is concerned with the classical technique of methylation and quantitative estimation of the derived methylated sugars; recent chromatographic methods have been fully utilised. Laminarin has been methylated by repeated sodium hydroxide-dimethyl sulphate treatments of the free polysaccharide. In a pilot experiment a product I having 44.4% methoxyl and $[\alpha]_D^{19}$ -6.5° was obtained after seven such treatments. Methanolysis of I and subsequent hydrolysis of the glucosides in the usual manner resulted in the production of a mixture of methylated glucoses which was examined by paper chromatography as applied to methylated sugars (20, 21). Three sugars were observed by this technique, two having R_G values identical with tetramethyl- and 2:4:6-trimethyl glucose and a third probably a dimethyl glucose having an R_G value of about 0.55. The presumed 2:4:6-trimethyl glucose appeared to be homogeneous and was the major component of the mixture indicating that the polysaccharide is made up wholely of 1:3-links.

Methylated laminarin II, $[\alpha]_D^{15}$ -6.5°, was obtained in 53% yield after seven dimethyl sulphate treatments followed by two treatments with silver oxide-methyl iodide, which, together with a final treatment with dimethyl sulphate, failed to raise the methoxyl content above 43.4%. A third methylation was carried out in an attempt to substitute the molecule fully and to fractionate the end product. After nine dimethyl sulphate

methylations the product was fractionated from chloroform solution by light petroleum giving three fractions:

IIIa.	8.75g.	-OMe 41.2%	Total yield from free polysaccharide, 60%.
IIIb.	6.25g.	" 43.8%	
IIIc.	0.75g.	" 37.5%	

Fraction IIIb. was twice methylated with Purdie's reagents but without significantly increasing the methoxyl content. Fractions IIIa. and IIIc. were combined and re-methylated three times with dimethyl sulphate and the product again fractionated giving two main fractions:

$IIIa_1$	3.49g.	-OMe 44.4%
$IIIa_2$	3.04g.	-OMe 43.6%.

It is likely that the introduction of further methyl groups would not be easy, if at all possible, and at this stage fractions $IIIa_1$, $IIIa_2$ and IIIb were taken to be fully methylated. The specific viscosities of these fractions were 0.316, 0.306 and 0.294/at 20° in m-cresol, respectively, showing that the methylated polysaccharide was substantially homogeneous.

Two methods of analysing the mixture of methylated glucoses produced on hydrolysis of the above methyl products were used, namely quantitative macro-separation by partition on a column of powdered cellulose (3), and micro-determination of each component after separation on a paper chromatogram (21). For an initial experiment methylated product II was methanolised with 2% methanolic-hydrogen chloride for 50 hours (constant rotation),

and neutralised with silver carbonate giving a non-reducing syrup of methyl glucosides (II). The free crystalline sugars were then obtained by hydrolysing syrup II for 4 hours with 2N hydrochloric acid on a boiling water bath and working up in the usual manner. The sugar mixture, dissolved in a little solvent, was introduced onto the top of a column 3.5 x 41cm. long of powdered cellulose (Whatman ashless filter tablets) previously prepared by elution with a solvent composed of 60% petroleum (b.p. 100°-120°), 40% butanol, saturated with water. Elution was commenced with the same solvent and 4ml. fractions collected, utilizing a fraction cutter of the type described by McGilvray (22). Fractions were examined by paper chromatography and those containing tetramethyl and trimethyl glucose appropriately collected together, evaporated and purified. On analysis the tetramethyl fraction was found to contain appreciable amounts of trimethyl methylglucoside and therefore it was re-hydrolysed and separated chromatographically on a small cellulose column, 1.6 x 37cm. From this separation pure tetramethyl glucose corresponding to 4.6% of the isolated products was obtained. The dimethyl and monomethyl fractions, eluted together with water, were unfortunately lost during isolation. This figure of 4.6% end-group corresponds to a repeating unit of about 22 residues, but this result is to be considered as provisional only since the number of operations

in the isolation of the end-group was large. The end-group was authenticated as 2:3:4:6-tetramethyl-D-glucose by recrystallisation from light petroleum giving white needles, m.p. 83-85° not depressed on admixture with an authentic sample. The needles analysed as the tetramethyl sugar and had $[\alpha]_D^{15}$ +83.3° (c,0.87 in water). The anilide had m.p. 134-6°, m.m.p. 135-6° with authentic tetramethyl glucose anilide, and gave a good analysis. The identity of the trimethyl glucose was fully established as the 2:4:6-D-derivative and its homogeneity shown by paper chromatography. On re-crystallisation from dry ether it had m.p. 124-6°, $[\alpha]_D^{16}$ +91.3° (initial)→ +75.5° (equil., c,1.03 in water) and gave good analytical figures. The lactone prepared in the usual manner had $[\alpha]_D^{17}$ +95°→+42.7° (6 hours constant, c,2.2 in water), and on treatment with methanolic ammonia gave a syrupy amide which could not be crystallised, $[\alpha]_D^{16}$ +35.0° (c,1.1 in acetone). The sugar readily gave a crystalline anilide m.p. 163-5°, $[\alpha]_D^{15}$ -81.0° (c,0.58 in methanol), which also analysed well. For 2:4:6-trimethyl glucose, Lake and Peat (23) give m.p. 115°, $[\alpha]_D^{20}$ +98.2→74.8°; lactone, $[\alpha]_D^{21}$ +96.0→39.0° (6 hours); amide (crystalline), m.p. 100, $[\alpha]_D^{20}$ +37.0°. Granichstädten and Percival (24) record m.p. 162-6°, $[\alpha]_D^{15}$ -133° for the anilide. The links in laminarin have therefore been proved to be exclusively 1:3 since no other trimethyl glucose other than the well-established 2:4:6-derivative could be detected.

The appearance of trimethyl methylglucoside in the tetramethyl fraction indicated incomplete hydrolysis of the methylglucoside mixture and attempts were made to avoid this in the hydrolyses of the glucosides obtained from methylated laminarin III. After 7 hours hydrolysis to constant rotation, and separation as before, the end-group fraction was again found to be contaminated. Re-hydrolysis and separation afforded 4.9% tetramethyl glucose corresponding to a repeating unit of 20.4 residues. The same phenomenon was observed even after 11 hours hydrolysis, when 5.13% end-group was finally obtained corresponding to 18.9 residues per repeating unit. The amount of trimethyl methylglucoside remaining after hydrolysis is small being about 2 or 3% of the total but its occurance means that in the normal hydrolysis of methyl glucosides with mineral acid, an equilibrium must be set up and the reaction does not proceed to completion as is usually supposed. Similar events have been experienced by other workers in these laboratories when the reaction has been carried out under reflux or in a sealed tube; the non-removal of methanol as a hydrolytic product probably prevents completion:

$$C_9H_{17}O_5(OCH_3)+H_2O \overset{H^+}{\rightleftharpoons} C_9H_{18}O_6+CH_3OH.$$

It is probable that the tetramethyl methylglucoside arising from end-groups is also not completely hydrolysed and that some is lost in the eluate preceding that first collected. The amount

will clearly be small, effecting a decrease of perhaps half a residue in the above/end-group figures.

In the last separation, the dimethyl fraction was slowly eluted with a 50% petroleum, 50% butanol mixture. No difference in the nature of this fraction could be detected during elution, the R_G value of the eluate residue remaining constant. Altogether this fraction, isolated as a glass, accounted for 7.8% of the total sugars eluted. By elution with water, about 1% of a mixture of monomethyl glucoses and glucose itself was obtained, but these materials were not considered to be structurally significant.

It was found that the mixture of glucoses obtained after 7 hours hydrolysis of glucosides III could only be analysed satisfactorily by the quantitative paper chromatographical method of Hirst, Hough and Jones (21), when it had been rendered rigorously ion-free. Removal of ions was effected by ion-exchange resins when reproducible figures of $5.4 \pm 0.3\%$ and $8.3 \pm 0.5\%$ for tetramethyl and dimethyl glucose respectively were given. This corresponds to a repeating unit of 19 ± 1 residues and agrees reasonably well with the previous analyses.

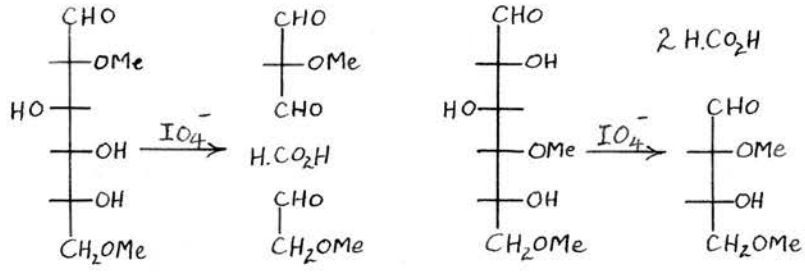
Methylation data thus reveal a molecule having about 5% end-group and the possibility of 8% branched positions which give rise to the dimethyl fraction. Theoretically, the amount of branching cannot exceed the amount of end-group if the polysaccharide links are all of the normal glycosidic type.

Also, in a molecule of about 40 residues size as laminarin would appear to be from its reducing power, the amount of dimethyl glucoses derived from branching can only be about 2.5% assuming a repeating unit of 20 residues (see p.58). The "extra" dimethyl glucose may be either the result of undermethylation of the polysaccharide or partial demethylation during hydrolysis. The latter particularly concerns the demethylation of the 2:4:6-trimethyl glucose residue to dimethyl glucoses and this effect was investigated on a sample of chromatographically pure sugar. The methylglucoside was prepared under the same conditions used for methanolising the methylated polysaccharide, and then hydrolysed with 2N hydrochloric acid at boiling-water temperature. A definite amount of demethylation occured with this treatment and the mixture of 2:4:6-trimethyl glucose and dimethyl glucoses was analysed by paper chromatography and by bulk separation on cellulose. The former method with a 7-hour hydrolysis gave dimethyl glucose amounting to 4.7% of the total mixture whilst bulk separation of an 11-hour hydrolysis gave as much as 8.7%. Such marked sensitivity to hydrolytic conditions is quite unusual with methylated sugars in general, but may be a particular propensity of 2:4:6-trimethyl glucose or a result of the conditions employed in this case. Bell (25) has noted demethylation with 2:3:6-trimethyl glucose to the extent of about 2% when the pure methylglucoside was hydrolysed for 5 hours

with a mixture of glacial acetic acid and approximately N hydrochloric acid . The present observations of demethylation make it possible to explain the existence of all the dimethyl fraction on this basis, and the possibility of branched positions in the polysaccharide is left in doubt. The origination of the dimethyl fraction through demethylation receives some support from the similarity of the dimethyl fractions from the methylated laminarin and demethylation, respectively. These were both glasses which partially crystallised slowly and had 27.7% methoxyl (a dimethyl hexose contains 29.8% methoxyl) and $[\alpha]_D^{16}$ +68,6° and +67.8°, respectively. It was noted that both gave only the same single spot on the paper chromatogram even when the distance travelled down the paper was over 30cm., but that both spots appeared to be composite, having a pink "body" and a light brown "tail" towards the slow portion, when developed with aniline oxalate. Oxidation with periodate (25) gave no formaldehyde showing that the 6-position is fully substituted and reducing the possible dimethyl glucoses to the 2:6- and 4:6-derivatives. Through the kindness of Dr. D. T. Bell, pure specimens of these derivatives were obtained and on examination by paper chromatography were shown to have almost the same R_G values as each other and as the dimethyl fractions referred to above. However, the 2:6- and 4:6-dimethyl glucose gave quite different colours with aniline oxalate, namely distinctly pink and brown, respectively.

2:4-dimethyl glucose has an appreciably larger R_G value (22) and gives a pink colour. Furthermore, by crystallisation from ethyl acetate of the dimethyl fraction from methylated laminarin, what appears to be pure 4:6-dimethyl glucose has been obtained, m.p. 159-162°, m.m.p. with authentic 4:6-dimethyl glucose (m.p. 154-9°) 155-9°. The 2:6-derivative has only been obtained as a glass (26).

It is very likely, therefore, that the dimethyl fractions are mixtures of 2:6- and 4:6-dimethyl glucoses. The composition of these mixtures was approximately determined by oxidising them with periodate and comparing the equilibrium rotations with that of pure 4:6-dimethyl glucose oxidised under the same conditions. 2:6-dimethyl glucose (III) on oxidation will give only optically inactive products, whilst the 4:6-compound (IV) will give weakly active 2:4-dimethyl-D-erythrose (V):



III IV V

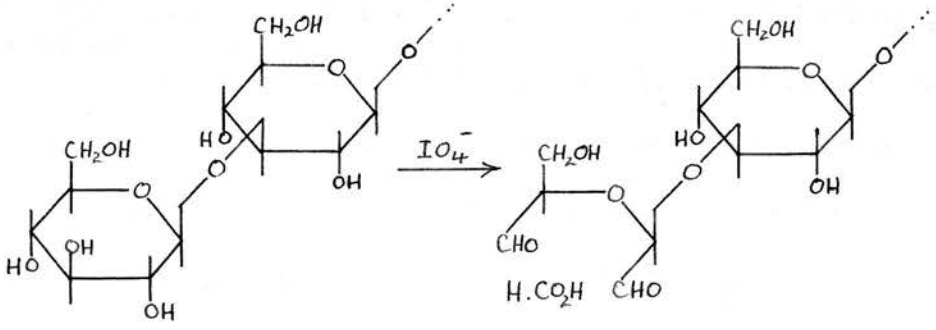
By this method the dimethyl fractions from methylated laminarin and demethylation were found to contain 49% and 54% 4:6-dimethyl

glucose, respectively. Demethylation of 2:4:6-trimethyl glucose might be expected to give equimolecular amounts of 2:4-, 2:6- and 4:6-dimethyl glucoses, but from these experiments the methyl group on position 6 seems particularly stable. The approximately equivalent compositions of the two dimethyl fractions thus lends support to the possibility that the whole of the dimethyl glucoses isolated from methylated laminarin results from demethylation.

The structural detail from these methylation studies has been amplified by an examination of the action of periodate on laminarin. By the action of periodic acid on laminarin, Barry (7) and Dillon (27) have shown that only terminal residues are oxidised, and by cautious removal of these oxidised residues the greater proportion of the molecule could be recovered unchanged. This observation is in accordance with the view that the polysaccharide is 1:3-linked. In the present instance, interest lay not in stepwise degradation but in an estimation of the end-groups in the molecule by determination of the formic acid they produce. This has been achieved with a number of polysaccharides (28, 29, 30) using the sparingly soluble potassium metaperiodate, sufficient of which remains in aqueous solution to effect quantitative oxidation in a reasonable time whilst over-oxidation is reduced satisfactorily. Laminarin was oxidised under the same conditions and estimates of the formic acid liberated were made periodically; the original sample,

fraction A and fraction B were all examined since the method affords evidence of the homogeneity of the polysaccharide in that it would detect differences in chain-length and degree of branching. The results are shown in the accompanying graph III (p. 50). Oxidation was normally conducted in daylight but one experiment was carried out in complete darkness following the observation of Head (31) that the considerable over-oxidation of cellulose which occurs with sodium periodate in daylight can be eliminated by oxidation in the dark. On examination of the graph, however, it appears that light has little noticable effect on the reaction with laminarin.

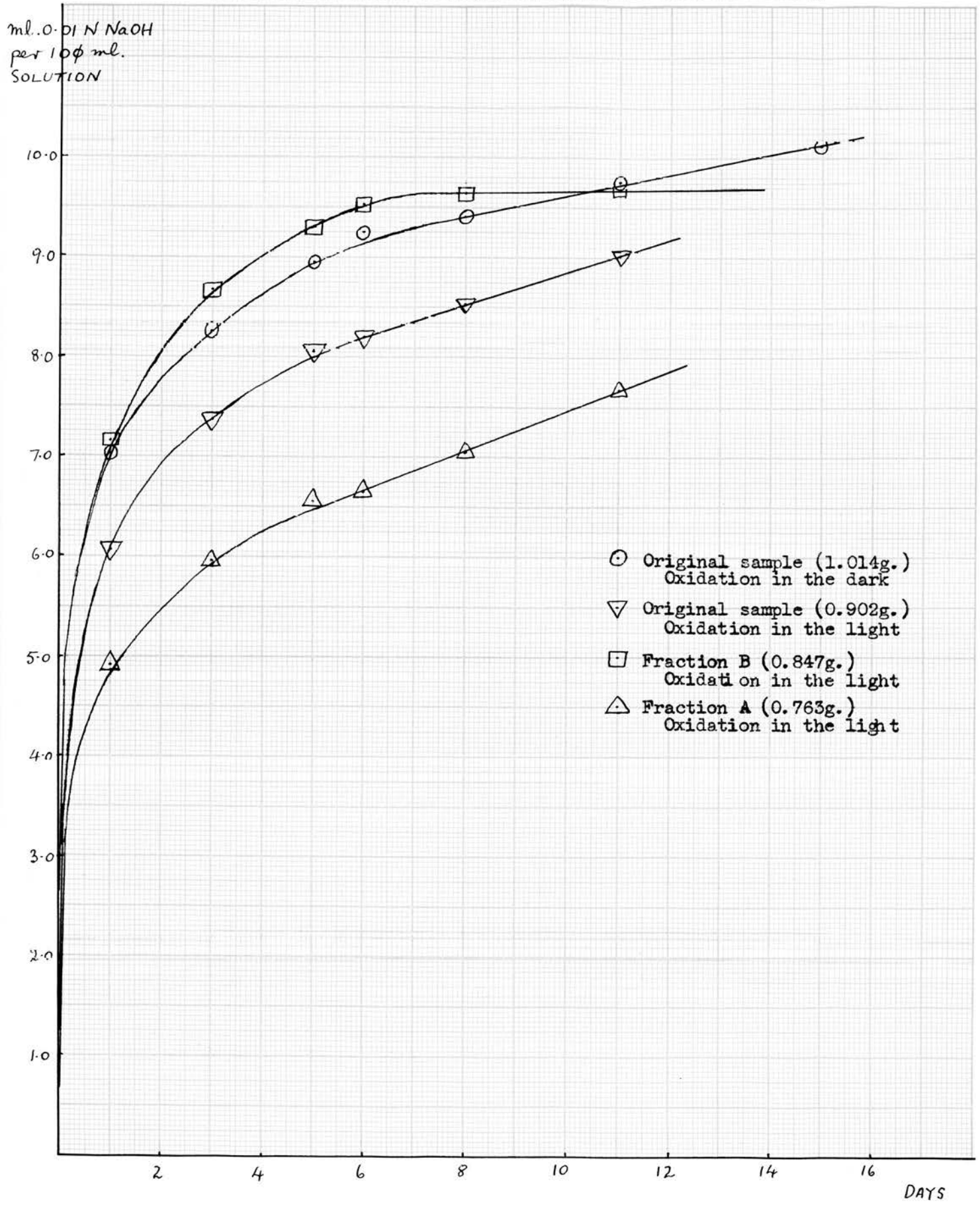
If the theoretical reaction is considered, the non-reducing end-groups of laminarin which are normal, will each give rise to 1 molecule of formic acid:

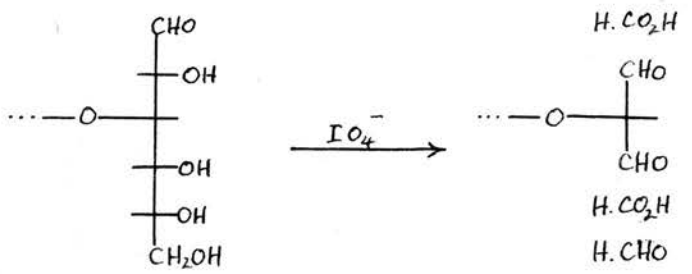


With reducing end-groups the reaction is not so well-defined since this group may be considered to exist as an equilibrium mixture of the aldehydo- and pyranosido-forms. The reaction with the aldehydo-form (VI) will be unequivocal, producing two molecules of formic acid:

GRAPH III

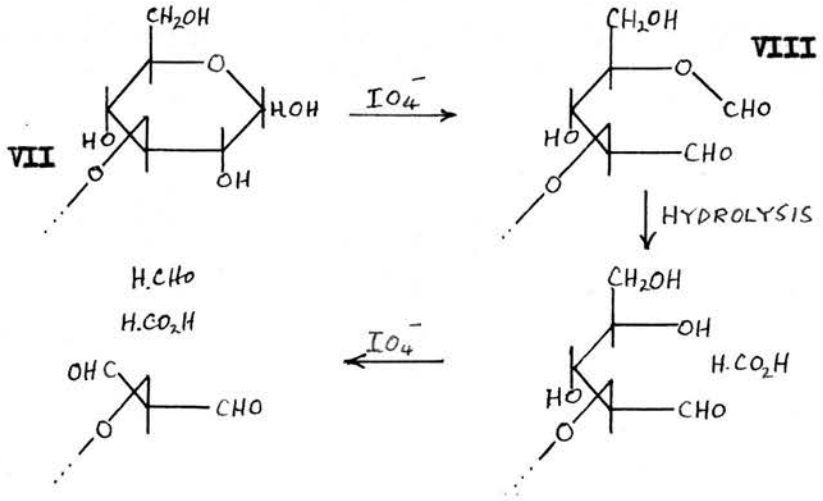
Liberation of Formic Acid on KIO_4 Oxidation of Laminarin.



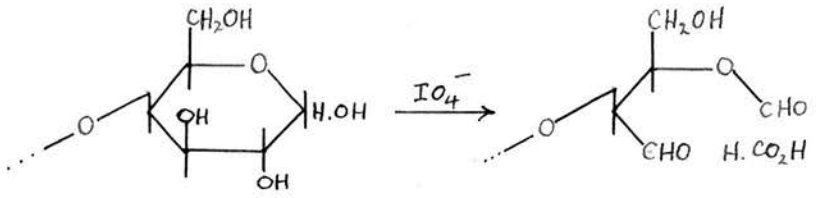


VI

The initial reaction with the ring form (VII) is the fission of the bond between C_1 and C_2 in which no formic acid is liberated, and only if hydrolysis of the semi-acetal structure (VIII) so formed occurs is formic acid produced as with the aldehydo-form:



A complete reaction of all reducing end-groups cannot be expected, then, unless such a hydrolysis is ensured. Brown, Dunstan, Halsall, Hirst and Jones (28,30) and Potter and Hassid (32) consider that oxidation of reducing residues in starches is complete under normal conditions of oxidation, that is pH4, but Meyer and Rathgeb (33) consider that only one molecule of formic acid arises from these residues:

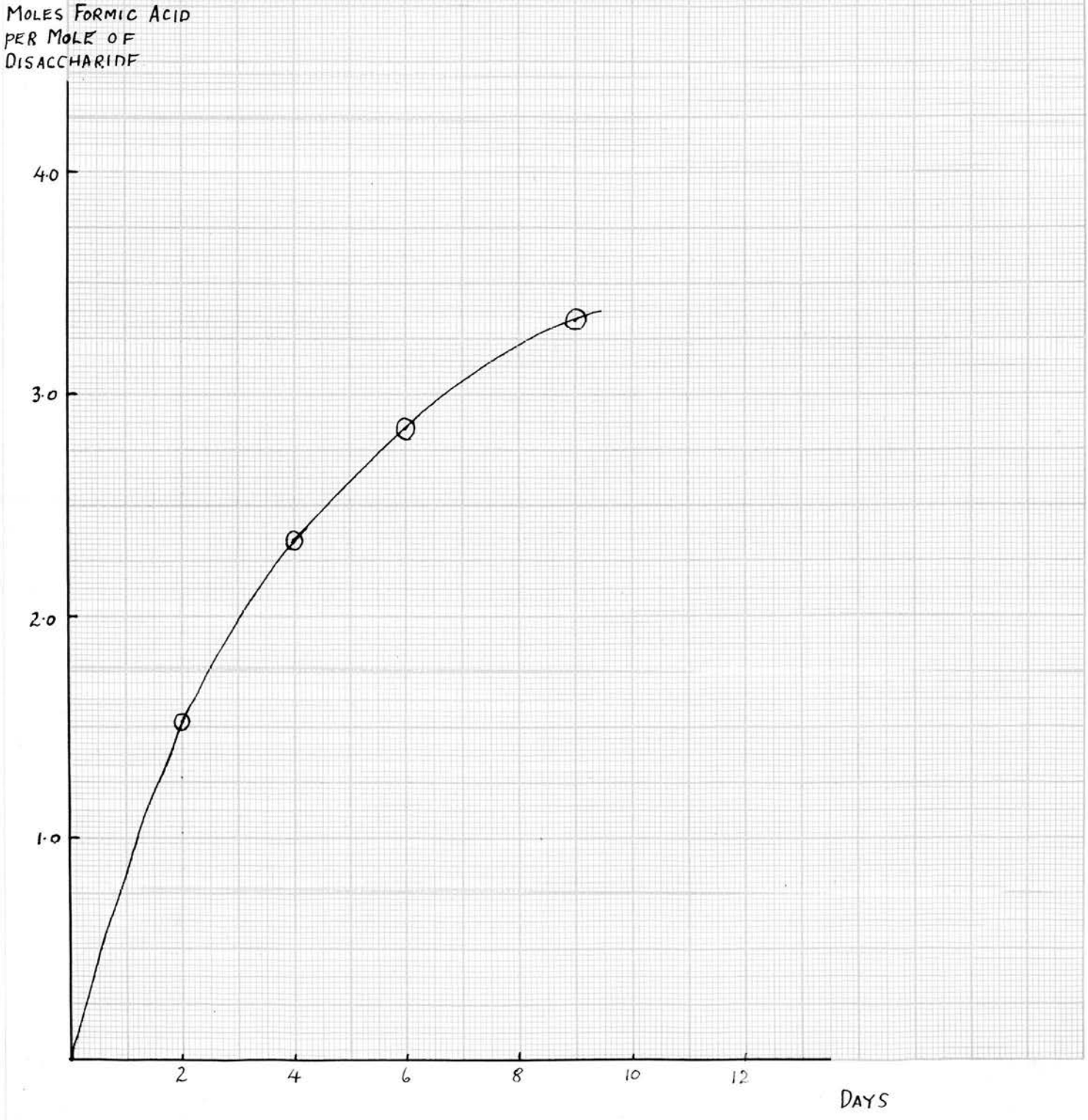


It must be pointed out, however, that the latter authors base their considerations on the results of rapid initial oxidation of lactose and maltose as model molecules, and although this oxidation appears to furnish two molecules of formic acid per molecule of disaccharide, continued reaction after about 20 hours produces further appreciable amounts of formic acid and it is from these longer time intervals that the results on polysaccharides are obtained. Oxidation of laminarin was conducted over a period of several days and this fact together with the large amounts of formic acid encountered, high consumption of periodate, liberation of formaldehyde and apparent symptoms of over-oxidation, probably infer that intermediate hydrolysis occurs.

This conclusion is supported by the extensive oxidation by potassium periodate of laminaribiose at pH8 (see p.112). The accompanying graph (IV) (p.53) shows that two and three moles of formic acid, or rather titratable acid, are liberated per mole of disaccharide after three and seven days oxidation, respectively. Periodic acid oxidation of 1 mole of 1:4-linked disaccharides yields two moles of formaldehyde according to Ahlborg (34) and similar oxidations at pH7.5 performed by Jeanloz (35) give only slightly modified results. These

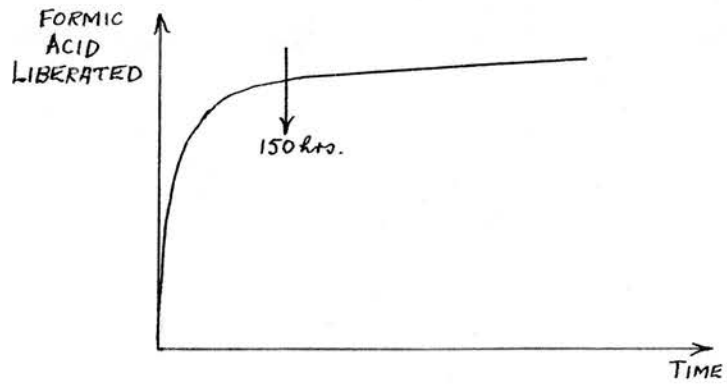
GRAPH IV

Liberation of Formic Acid on KIO_4 Oxidation of Laminaribiose.



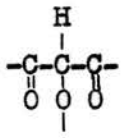
observations clearly indicate the more or less complete oxidation of the reducing residue and although the precise course of periodate oxidation of these residues depends largely upon conditions of concentration, pH, etc., it is considered probable that the same reaction occurs at the reducing end-group of laminarin.

Returning to a consideration of the experimental results, a rapid initial reaction which becomes slower as oxidation of the end-groups becomes more complete, is apparent in all four cases. After about five days, formic acid continues to be liberated appreciably at a linear rate from the original sample and fraction A, whilst the liberation from fraction B appears to cease. With the hardly- or non-reducing polysaccharides glycogen and amylopectin, the rate of liberation is at first rapid and then slackens off during 2-6 days to a very low value:

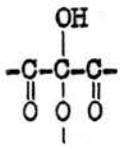


Oxidation is taken to be complete in respect of end-groups after 150 hours, that is the time required to oxidise quantitatively β-methylmaltoside under the same conditions (29, 30). With laminarin it is difficult to decide at which stage the end-group

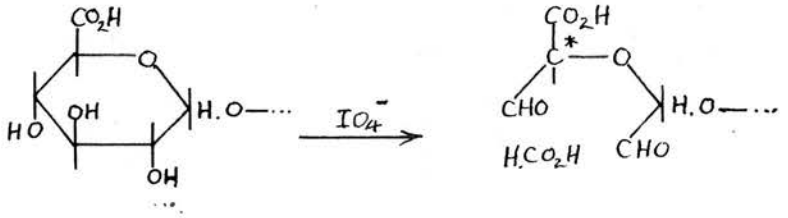
oxidation is complete because of the continued rise in acid content of the solution. This rise together with the appearance of iodine which accompanies it, are considered to be symptomatic of the well-known phenomenon of over-oxidation. It has been noted (28, 29, 32) that oxidised molecules possessing the system:



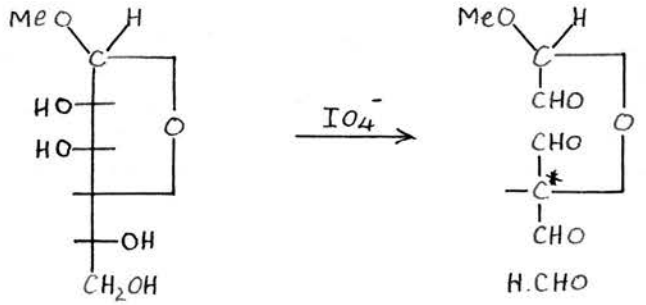
are susceptible to the further action of periodate even under controlled conditions. The activated H atom on the central C atom is probably oxidised to -OH and the system:



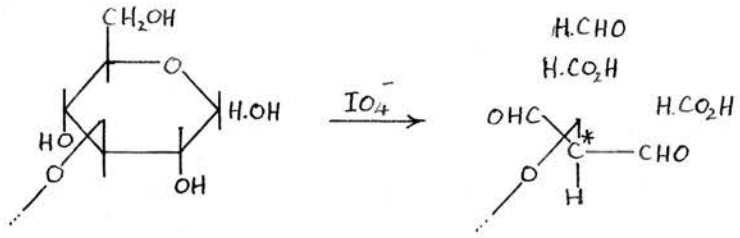
then gives rise to further amounts of acidic materials, including formic acid. Thus polysaccharides containing terminal uronic acid residues, being initially oxidised as follows:



are further oxidised at C* (28). Similar behaviour is noted with the oxidation product from glycosides of uronic acids (36) and from α-methylmannofuranoside (29):



Potter and Hassid (32), using sodium metaperiodate in aqueous solution at 2°C, find over-oxidation with the reducing carbohydrate maltose, and with starch amyloses where, relative to amylopectin fractions, the proportion of reducing end-groups is high. It is probable that an analogous system is produced at the reducing end-group of laminarin (see p.51, VII):

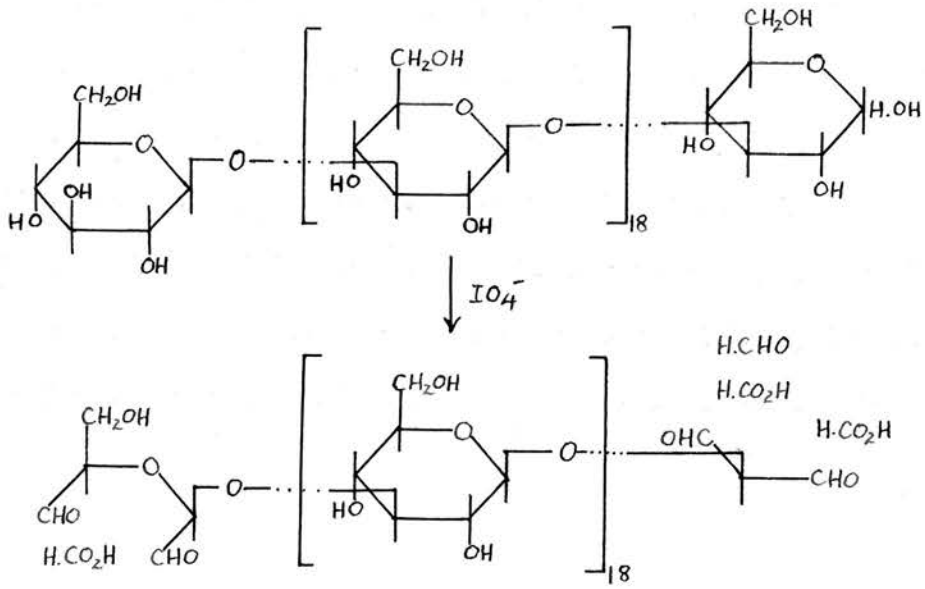


and since laminarin is fairly reducing, the further liberation of acidic materials may be expected to be appreciable. The linear portions of the curves for the original sample and fraction A are therefore considered to be due to secondary oxidative changes occuring after about 6 days.

For the original sample, the titre at four and five days corresponds to the liberation of 1 mole of formic acid per 7.0 and 6.7 anhydro-glucose residues, respectively, in the light and 7.1 and 6.8 in the dark. For fraction A the figures for oxidation in daylight are 7.3 and 7.0, respectively, thus it is probable that these two samples are very similar, if not identical. Fraction B is different in that the curve flattens

and oxidation appears almost to cease after about 7 days when the liberation corresponds to 1 mole per 5.3 residues. A sample of soluble laminarin kindly given by Dr. V. C. Barry gave a figure of 3.8 residues after 4 days. It is not yet clear whether these differences are due to the solubility of the samples or to the fact that actual fractionation has been achieved, and pending more information about the properties of alcohol-precipitated laminarins, their study by periodate oxidation has been discontinued.

The high yields of formic acid from normal laminarin denote either a high degree of branching, when large numbers of terminal residues will be available for attack, or a straight-chain structure of short length. Calculation shows that the low proportion of end-groups, as determined by methylation, is incompatible with the former possibility, and that the only structure which will furnish about 5% end-group and liberate 1 mole of formic acid per 7 residues is a straight chain about 20 residues in length



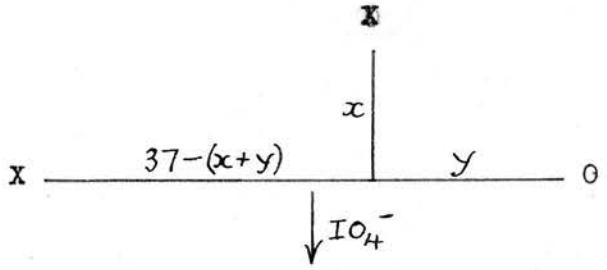
i.e. 3 $H.CO_2H$ are produced from 20 residues.

Methylation would ideally give 5% end-group and no dimethyl glucoses.

A branched type of structure the molecular size of which agrees with the results of hypoiodite oxidation is shown diagramatically below:

X - non-reducing end-groups, furnishing 1 mole of formic acid on periodate oxidation.

O - reducing end-group - furnishing 2 moles of formic acid on periodate oxidation.



4 moles of formic acid per 40 residues.

Methylation would ideally give 5% end-group and 2,5% dimethyl glucose.

The former structure need not conflict with the isolation of appreciable amounts of dimethyl glucose after methylation since, as has been observed above, these may be explained by demethylation and undermethylation, but it certainly does not agree with the reducing power of laminarin as measured by hypoiodite oxidation. The latter normally gives high results and the molecular size of 40-45 residues would be expected to be

a minimal figure, but it is possible that direct measurement of reducing power is misleading in this case. The proximity of 1:3-links has no obvious diminishing effect on the reducing power, laminaribiose giving almost quantitative oxidation with hypoiodite. Only the reducing end-groups of 1:3- and 1:4-linked polysaccharides will yield formaldehyde on periodate oxidation by virtue of their oxidisable primary hydroxyl group, and Caldwell and Hixon (37) have found that the amount of formaldehyde liberated from starch dextrins can be directly correlated to reducing power. However, similar estimates with laminarin showed that after 4 days oxidation 1 mole of formaldehyde was liberated per 27 residues, which is in much closer agreement with the molecular size required by the previous periodate oxidations.

Estimates of the total consumption of periodate during oxidation also support the formulation of a smaller molecule. For each C-C bond broken in the oxidation, one molecule of periodate is reduced quantitatively to iodate (38), therefore non-reducing end-groups will consume 2 moles and reducing end-groups (for complete oxidation) 3 moles of periodate. Thus a straight chain of about 20 1:3-linked residues would consume a total of 1 mole of periodate per 4 residues, whilst a singly branched structure of 40 residues size would consume 1 mole per 6 residues. Actually, the consumption after 5 days oxidation under conditions similar to those employed for end-group assay,

was found to be 1 mole per 3.9 residues for fraction A (oxidation in daylight) and per 3.6 residues for the original sample (in the dark). A strict interpretation of these figures as meaning consumption of periodate due to oxidation of end-groups is, however, impossible in view of the fairly rapid additional or over-oxidation.

The results of the two physico-chemical measurements so far undertaken indicate that the molecular weight is less than about 3000, corresponding to about 20 anhydro-residues. From the specific viscosity of methylated laminarin an approximate idea of the molecular size may be obtained, though in the absence of any definite knowledge of the molecular shape it is impossible to make a valid application of the Staudinger equation (39):

$$M = \frac{\eta_{sp}}{c.K_m}$$

where: M = molecular weight

c = number of moles of unit residue per litre

K_m = molar viscosity constant.

Hirst and Young (40) observe a value of K_m of $1.3.10^{-4}$ for starches of molecular weight greater than 20,000 and of about 10^{-3} for less than 20,000. The evidence indicates a molecular weight of laminarin much less than 20,000 and therefore $K_m = 10^{-3}$ is used in conjunction with the η_{sp} of methylated laminarin III giving an apparent molecular weight of 2300-2600 for laminarin, that is 14-16 residues. Using $K_m = 1.2.10^{-3}$ for methyl cellulose,

slightly lower figures result. The micro-isopiestic method of determining molecular weights devised by Barger (41) has recently been adapted for the study of the molecular weights of starch and dextrin nitrates (42) and methylated laminarin has now been examined by the same technique. An approximate result of 2100-2750 or 13-17 residues was obtained but could not be confirmed with acetylated laminarin.

On the whole the results of periodate oxidation suggest a straight-chain molecule of 20-21 residues length and this is substantiated by methylation data which reveal a repeating unit of 19-20 residues. The only conflicting evidence is that afforded by measurements of reducing-power which appear to give higher results of about 40 residues with hypoiodite. A reason for the divergency of reducing power cannot be sought in the mere presence of 1:3-links since laminaribiose behaves normally. It may be that some modification of the polysaccharide reducing group could explain all the results though it is difficult to visualise the form it would take. The reducing group is the most sensitive part of the molecule and some of these groups may be oxidised in the plant or during isolation. Also the possibility of glycosidic or semi-acetal groups must not be overlooked and it is as well to remember that discrepancies in reducing power are not unknown in polysaccharide chemistry. As yet it would be improper to advance a structure of laminarin which is definite in all its detail

but it is clear that the bulk of the evidence points to a straight-chain molecule composed of 20 1:3-linked anhydro-glucose residues in the β-configuration. It is hoped that when more accurate determinations of molecular weight are forthcoming, particularly from osmometry, a conclusive answer can be given.

EXPERIMENTAL

Extraction of laminarin.

The method employed by the Scottish Seaweed Research Association follows that of Barry (1) and has been described in the Discussion (p.26).

Purification of laminarin.

The original sample (60g., 10% moisture) was dissolved in distilled water (1L.) at 50° giving a slightly brown-coloured solution which was allowed to deposit for 72 hours. The white deposit of laminarin was removed on a sintered funnel and re-deposited 3 times when the product (fraction A) showed negative tests for $SO_4^{=}$ and Cl^{-}.

Fraction A: wt. 40g. 24.0% moisture, 0.2% ash.

$[\alpha]_D^{15}$ -14.8° (c,0.78 in water).

The actual weight of fraction A (30.4g.) represents a 57% recovery.

The depositing solutions were combined (4L.), concentrated to 700ml. under reduced pressure at 50°, and allowed to stand for 72 hours. No deposition occurred, therefore the remaining laminarin (fraction B) was precipitated by addition of alcohol (3L.) and allowed to stand for 48 hours. It was removed by centrifuging and dried with ether and in a vacuum desiccator (concentrated sulphuric acid).

Fraction B: wt. 28g. 15.0% moisture, 0.2% ash.

$[\alpha]_D^{16}$ -12.9° (c,0.85 in water).

To observe optical rotations of insoluble laminarin, the suspension in water was warmed to dissolve, cooled and used before deposition commenced. Moisture contents were obtained by drying to constant weight at 60°C in vacuo over phosphorous pentoxide; ash contents were determined by incinerating the material with a few drops of concentrated sulphuric acid.

Hydrolysis of laminarin with dilute hydrochloric acid.

I Fraction A

0.251g. (dried in vacuo over phosphorous pentoxide at 60°C for 2 days) were heated with 0.85 N hydrochloric acid (10mls.) on a boiling-water bath and the optical rotation of cooled portions of the hydrolysate observed periodically in a 2dm. tube:

α°	-0.67	+0.91	+2.13	+2.55	+2.73	+2.80	+2.80
hrs.: mins.	0:10	0:30	1:00	1:30	2:00	2:30	3:30

Assuming the reaction: $(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6$

$[\alpha]_D$ = +50.4°, which represents 96.0% glucose.

The hydrolysate was neutralised with 2N sodium hydroxide (methyl orange) and carefully made up to 25 mls. with distilled water. The glucose in 5ml. portions of the solution was then estimated by hypoiodite oxidation (5ml. sodium hydroxide and 15ml. 0.1 N iodine solution added; allowed to stand 4 hours).

5ml. solution consumed 5.79 and 5.84ml. 0.1 N iodine.

≡ 52.3mg. glucose (average), which represents 94.5% glucose in the original hydrolysate.

II Original sample.

0.248g. (dry) were hydrolysed as above.

5ml. solution consumed 5.70 and 5.72ml. 0.1 N iodine.

≡ 5.14mg. glucose (average), which represents 93.3% glucose in the original hydrolysate.

Examination of laminarin hydrolysates by paper chromatography.

0.25g. quantities of the original sample and fraction A were hydrolysed with 25ml. 2N sulphuric acid for 4 hours at boiling-water temperature and the cooled hydrolysates neutralised with barium carbonate. After centrifuging, the clear solutions were examined on a filter-paper strip chromatogram with a glucose control. The mobile and stationary phases used in this and subsequent paper chromatography (unless otherwise stated) were the butanol and aqueous layers respectively, from a mixture of 50% n-butanol, 10% ethanol and 40% water, shaken intimately together. After running for 50 hours the paper was developed with ammoniacal silver nitrate solution when no reducing substance other than glucose could be detected in the hydrolysates.

Early in the work in this thesis it was found more convenient and that clearer chromatograms were obtained when a saturated aqueous solution of aniline oxalate was used as the developing reagent (43), and all paper chromatograms mentioned hereafter have been developed with this reagent. It gives distinct pink spots on an almost white background with aldo-hexoses, and with

most glucose oligosaccharides and methyl derivatives.

Partial hydrolysis of laminarin and separation of the hydrolysate components on a column of powdered cellulose.

Control experiment.

Laminarin (0.4g.) and N oxalic acid (10ml.) were heated under reflux on a boiling-water bath and small portions removed after 2, 4, 6, 8, 9 and 10 hours heating. These portions were neutralised with chalk, centrifuged and examined by paper chromatography. Laminaribiose developed as a spot having an R_F value about 0.4 that of glucose (butanol/ethanol/water) and the colour intensity of the spot was at a maximum after 6-8 hours hydrolysis.

Chromatograms of a 7-hour hydrolysate, when run for long periods (72 hours) showed the presence of a discrete spot, presumably due to a trisaccharide, having an R_F value about one-quarter that of glucose. Separation of the components of the hydrolysate was more marked when a faster-moving solvent (benzene 10 parts by volume, n-butanol 50 parts, pyridine 30 parts and water 30 parts) was used. With this solvent (48 hours) laminaribiose had R_F about 0.76 that of glucose, whilst two spots having R_F values of 0.50 and 0.32 that of glucose developed in the region where trisaccharides would be expected. The leading spot (0.50) which was much more intense than the other (0.32) probably arises from a laminaritriose.

Bulk experiment.

Laminarin (25g.) and N oxalic acid (750ml.) were heated on a

boiling-water bath for 7 hours and the solution cooled and neutralised with chalk. The solution after filtration was heated at 90°C for ½ hour to decompose any calcium bicarbonate, again filtered and evaporated under reduced pressure to 150ml. Charcoal was then added to decolorise and the filtrate concentrated to a thick syrup. Paper chromatography of this syrup showed the presence of much glucose and disaccharide.

Attempted removal of the glucose by yeast fermentation.

Half the above quantity of syrup was dissolved in water (300ml.) and incubated with washed yeast cells (ca.13g.) for 24 hours at 38°. The solution after filtration still contained glucose, therefore fermentation was continued for a further 24 hours. The suspension of yeast was then filtered with the aid of "FilterCel" and the filtrate concentrated to a clear glass, which contained no glucose and very little disaccharide (chromatogram). In addition, only an amorphous osazone could be obtained on treatment of the glass with phenylhydrazine, whereas laminaribiose readily gives a crystalline osazone (5).

Separation of the hydrolysate components on a cellulose column.

The general procedure follows that of Hough, Jones and Wadman (3).

The syrupy hydrolysate (3,4g.) was allowed to soak into the top of a cellulose column (powdered Whatman ashless filter

tablets; column dimensions, 3.5 x 41cm.) prepared by the percolation of half-saturated aqueous butanol, and elution commenced with the same solvent. It was found that water separated out of fully saturated and three-quarter saturated butanol if these were used for elution and half-saturation had to be resorted to despite the longer time of sugar elution. Because of this slow elution no attempt was made to collect small fractions of eluate and 500ml. lots of solvent were collected. After $2\frac{1}{2}$ litres the eluate was found to be reducing and evaporation of the solvent showed that only glucose was present. Continued elution, evaporation and examination of the residues by paper chromatography resulted in the following separation:

Next 1500ml. - glucose only

2000ml. - no sugar

4500ml. - laminaribiose

Changed to 60% - saturated butanol

Next 8000ml. - no sugar

4000ml. - impure trisaccharide.

In all 587mg. of laminaribiose were obtained as a colourless deliquescent glass after the appropriate residues had been de-colourised and degreased by treatment with a little charcoal. On hypoiodite oxidation 5.01mg. of the glass consumed 2.71ml. 0.01N iodine, whence the disaccharide content is 92.8%. The hydrolysate thus contains about 15% laminaribiose.

112g. of trisaccharide were obtained but this may not represent all that present in the hydrolysate since elution was not completed. The material, obtained as a glass, reduced Fehling's solution and contained small quantities of glucose and laminaribiose. It has not been further examined.

Laminaribiose.

Attempts were made to crystallise the above glass from absolute ethanol and glacial acetic acid. Crystallisation could not be readily effected with the latter but from ethanol a white, deliquescent microcrystalline powder separated. This lost water and coalesced to a glass at about 80°C and melted completely at 160-163°; a sharp melting-point cannot properly be recorded.

The glass had $[\alpha]_D^{''}$ +23.4 (15 mins.) → +19.0° (5 hrs., final) (c, 2.7 in water), and analysed as a disaccharide dihydrate:

Found : C 38.3% ; H 6.82%.

Calc. for $C_{12}H_{22}O_{11}.2.H_2O$: C 38.1% ; H 6.88%.

Laminaribiosazone.

Laminaribiose (80mg.) was heated on a boiling-water bath for 30 minutes with a saturated aqueous solution (2ml.) of phenylhydrazine hydrochloride and sodium acetate containing a small amount of sodium bisulphite. On slowly cooling the osazone separated and was recrystallised from hot water giving long yellow needles m.p. 200-202°. Yield 40mg. $[\alpha]_D^{14}$ -71.5°

(c,0.5 in absolute ethanol); analysis:

Found : C 52.3% ; 6.22% ; N 9.61%.

Calc. for $C_{24}H_{33}O_9N_4.2H_2O$: C 52.0% ; 6.15% ; N 10.1%.

Hypoiodite oxidation of laminaribiose.

To laminaribiose dihydrate (ca.5mg.) in water (5ml.) were added 0.1 N iodine solution (2ml.) and phosphate buffer (10ml., pH 11.4, c.f.ref.46) and the mixture allowed to stand for $3\frac{1}{2}$ hours. It was then acidified with 2 N sulphuric acid (10ml.) and titrated with 0.01 N thiosulphate.

Weight of disaccharide dihydrate (mg.)	Weight of disaccharide (mg.)	Uptake of iodine (ml.0.01 N)	% disaccharide found
5.21	4.71	2.86	~~87.0~~104.3
5.21	4.71	2.81	~~85.5~~102.5
5.01	4.54	2.71	~~85.5~~102.5

Attempted acetolysis of laminarin.

I. The method was similar to the well-known preparation of octaacetyl cellobiose (e.g.44).

Laminarin (5g., undried) was added in small quantities over half an hour to a well-cooled mixture of acetic anhydride (20ml.) and concentrated sulphuric acid (2.5ml.), the temperature being kept below 10°, and then allowed to stand at room temperature. The polysaccharide settled overnight to a somewhat sticky mass which was stirred daily into the solution over 11 days. After this time only 1/4 to 1/3 of the polysaccharide remained, whilst the solution had become fairly

viscous and brown-coloured. The whole was then poured into cold water (500ml.) and the white fibrous product so obtained was filtered off, washed free of $SO_4^=$ and dried in a vacuum desiccator (phosphorous pentoxide): Weight: 6.0g.

$[\alpha]_D^{16}$ -28.0° (c,0.89 in chloroform) ; acetyl content: 43.4%

The reducing power as determined by the method used for laminarin acetate (see below), indicated the presence of 1 reducing group per 3.7 anhydro-glucose units.

II. More vigorous conditions were adopted, following the method of Hibbert and Barsha (45) for octaacetyl cellobiose.

Laminarin (1g., undried) was added to a well-cooled mixture of acetic anhydride (4ml.) and concentrated sulphuric acid (0.1ml.) and then heated at 50° for 20 days. The solution became completely black in 2 days. After 20 days the solution, diluted with acetic acid, was poured into cold water (400 ml.) and allowed to stand overnight. The product, which was in very small yield and dark brown coloured, was isolated, and decolourised with charcoal in alcohol solution. Small quantities of fawn-coloured solid separated from alcohol on standing at 5°C, m.p. 55-65° (indefinite). None of these solids could be re-crystallised.

III. The duration of experiment II was reduced to 3 days in order to prevent the drastic degradation of 20 days heating.

The product on purification from alcohol gave a yellow gum which solidified on tiling, m.p. about 80° (indefinite).

IV. Laminarin (1g.) was added all at once to a mixture of acetic anhydride (4ml.) and concentrated sulphuric acid (0.1ml.), When it dissolved with evolution of much heat giving a light yellow solution. This solution was stirred and heated at 100° for 1 min., the colour then assuming a nut-brown shade, and poured immediately into water (300ml.). The product, worked up as before, again yielded non-crystalline solids from solidification of deposited gums.

Examination of the acetolysis products.

Small quantities of the above products were saponified overnight with methanol-saturated baryta. Barium was removed with 2N sulphuric acid and the neutral solutions examined by paper chromatography. Considerable amounts of glucose and small amounts of laminaribiose were present in all the products, but product IV appeared to contain the largest amount of disaccharide in proportion to the glucose.

Reducing power of laminarin.

Hypoiodite oxidation.

Quantities of laminarin (original sample) were dissolved in distilled water (10ml.) by heating gently. The solutions were immediately cooled under the tap to room temperature and approx. 0.1 N iodine in potassium iodide solution (10ml.) and approx. 0.2 N sodium hydroxide (8ml.) added to each. A blank was also made up without polysaccharide. Oxidation was allowed

to proceed at room temperature with occasional shaking, the solutions generally remaining clear throughout, though sometimes a slight milkiness appeared due to deposition of the laminarin. The iodine was then liberated by acidification (3ml. 2N sulphuric acid) and titrated with approx. 0.1 N thiosulphate.

Weight of polysaccharide (dry) g.	Time of oxidation (mins.)	I_2 uptake (ml.0.1047 N)	I_2 uptake in ml.0.1 N/ g. polysaccharide
0.184	35	0.55	3.13
0.274	35	0.80	3.06
0.361	35	1.01	3.20
0.573	20	1.50	3.06
0.936	30	2.80	3.13
0.945	45	2.80	3.10

Average: $\underline{3.11} \pm 0.05$.

$-CHO \equiv I_2$, and 1 mole of I_2 is consumed by $\frac{2}{3.11} .10^{-3}$ g. polysaccharide, whence the number of anhydro-glucose residues per -CHO is $\frac{2}{3.11}.\frac{10^{-3}}{162} = 40$.

Oxidation in buffered solution (pH 11.4).

Laminarin (original sample, dry) was dispersed as above in water (5ml.) and oxidised with 0.1 N iodine solution (5ml.) and a Na_2HPO_4-NaOH buffer (10ml., pH 11.4, c.f.ref.46) in "Quickfit" test-tubes, the stoppers of which were moistened with 10% potassium iodide solution. The reaction mixtures plus a blank were allowed to stand at room temperature, acidified (10ml. 2 N sulphuric acid) and titrated with 0.1 N thiosulphate.

Weight of polysaccharide (dry) g.	Time	I_2 uptake (ml.0.1 N)	No. of anhydro-residues per -CHO
0.1978	50 mins.	0.52	46.7
0.3016	18 hrs.	0.90	41.1
0.4016	100 mins.	1.04	47.5

Oxidation with bromine.

Bromine (2.3ml.) was added to a cold aqueous solution of laminarin (original sample, 1.5g. dry in 50ml. distilled water) and the mixture kept in the dark with occasional shaking for 15 days. At the end of this time the bromine was removed by aeration and the white suspension of oxidised laminarin filtered off, washed well with water and then dried with alcohol and ether. After drying in a vaccuum desiccator (phosphorous pentoxide) the free-funning powder weighed 1.14g.

0.5g. of this material required 18.80ml. 0.00944 N sodium hydroxide for neutralisation after boiling (phenolphthalein), which corresponds to 1-COOH group per 17.4 glucose residues. That this high figure was due to the presence of low molecular weight acidic fragments was shown by dialysing the remainder (0.64g.), when the weight fell to 0.37g.

0.3g. of this dialysed product (absolutely dry) required 4.02ml. 0.00952 N sodium hydroxide for neutralisation, corresponding to 1-COOH group per 48.4 glucose residues.

Molecular weight by reduction of 3:5-dinitrosalicylic acid.

(Meyer, Noelting and Bernfeld (10))

Standardisation curve of laminaribiose.

Solution A. 1.5% aqueous 3:5-dinitrosalicylic acid.

" B. 6N sodium hydroxide.

The curve was prepared by heating 2ml. of carbohydrate solution of various concentrations with 1ml. each of water and solutions A and B for 30 minutes at 65°, cooling, diluting exactly to 25ml. and comparing the intensity of colour with a blank treated under the same conditions but omitting the carbohydrate (an extra 2ml. water added instead). A Spekker spectrophometer was employed in the comparison using an Ilford green filter 604.

mg. laminaribiose	Micromoles	Drum reading (4cm. cell)
1.86	5.44	1.01, 1.01
1.49	4.36	0.745, 0.735
1.12	3.28	0.434, 0.434
0.96	2.81	0.357, 0.362
0.74	2.16	0.197, 0.193
0.50	1.46	0.115, 0.120
0.38	1.11	0.046, 0.049.

Reaction with laminarin.

2ml. of 0.508% and 0.238% aqueous solutions (solubilised by warming) were heated as above with 1ml. each of water, and solutions A and B, and compared after cooling and making up to 25ml. with a blank in which solution A had been added only after

cooling. This is necessary since the polysaccharide itself gives a slightly yellow colour on heating with strong alkali.

The drum readings using 4cm. cells were 0.70 and 0.21 for the 0.508% and 0.238% solutions, respectively. From the standard curve, the weights of laminaribiose having the same absorption are 1.46 and 0.74mg., respectively, whence:

Degree of polymerisation (2ml. of 0.508%) = $\frac{2.\ 1.016.10}{1.46}$ = 13.9.

(2ml. of 0.238%) = $\frac{2.\ 0.476.10}{0.74}$ = 12.9.

Reaction curve of cellobiose.

mg. cellobiose	Micromoles	Drum reading (4cm. cell)
2.12	6.18	0.99
1.91	5.57	0.875, 0.880
1.70	4.96	0.695, 0.695
1.48	4.32	0.556, 0.560
1.27	3.71	0.446, 0.448
0.85	2.48	0.225, 0.226
0.42	1.23	0.094, 0.094

Reaction curve of maltose.

mg. maltose	Micromoles	Drum reading (4cm. cell)
2.02	5.89	1.10
1.81	5.28	0.88, 0.88
1.61	4.69	0.728, 0.730
1.21	3.53	0.418, 0.420
0.81	2.34	0.183, 0.184

Reaction curve of glucose.

mg. glucose	Micromoles	Drum reading (4cm. cell)
1.00	5.56	0.523, 0.524
0.90	5.00	0.402, 0.404
0.80	4.44	0.321, 0.321
0.70	3.89	0.273, 0.273
0.60	3.33	0.225, 0.225
0.40	2.22	0.140, 0.140
0.20	1.11	0.029, 0.027

Reaction curve of 3-methylglucose.

mg. 3-methylglucose	Micromoles	Drum reading (4cm. cell)
1.05	5.42	0.214, 0.211
0.95	4.90	0.191, 0.193
0.84	4.33	0.177, 0.178, 0.169
0.73	3.77	0.155, 0.157, 0.139
0.63	3.25	0.112, 0.116
0.52	2.68	0.095, 0.095
0.42	2.17	0.050, 0.053, 0.062

Reaction curve of 4-methylglucose.

mg. 4-methylglucose	Micromoles	Drum reading (4cm. cell)
1.00	5.17	0.172, 0.173
0.90	4.64	0.165, 0.165
0.80	4.13	0.138, 0.136
0.70	3.61	0.118, 0.120
0.60	3.09	0.096, 0.096
0.40	2.12	0.047, 0.048.

Reaction with 2:4:6-trimethyl glucose.

mg. 2:4:6-trimethyl glucose	Micromoles	Drum reading (4cm. cell)
2.0	8.63	0.005
1.6	6.90	0.000.

Acetylation of laminarin.

I. Using Barnett's reagents.

Laminarin (5g.) was dissolved in hot water (75ml.) and precipitated with alcohol (650ml.), filtered, washed with hot alcohol and ether and finally dried in a vacuum desiccator.

This product (4.15g.) was mixed with glacial acetic acid (30ml.) containing chlorine (1.27g.) and left for 30 minutes with occasional shaking. Acetic anhydride (50ml.) containing sulphur dioxide (1.35g.) was then added and the mixture shaken. Heat was evolved and the laminarin almost completely dissolved in a few minutes. After filtering off a small quantity of insoluble material, the filtrate was poured into water (600ml.) and the fine white precipitate which deposited was allowed to stand in water for 48 hours. The acetyl product (I) was filtered off, washed well with water, dried with alcohol, ether and finally in a vacuum desiccator (concentrated sulphuric acid).

Weight: 5.6g. i.e. 68% theory.

$[\alpha]_D^{16}$ -58.8° (c,1.1 in chloroform).

The acetyl content of this and the following acetyl products was determined by dissolving about 0.1g. in A.R. acetone (25ml.) and saponifying with approximately 0.1N potassium hydroxide (25ml.), which did not precipitate the compound out of solution. The mixture and a blank were allowed to stand at room temperature for 3 hours and then titrated with 0.1N hydrochloric acid (phenolphthalein).

0.0944g. acetyl product I required 11.00ml. 0.085N hydrochloric acid

Blank " 22.25ml. " " "

Whence: % acetyl (CH_3CO-) is 43.9%.

II. By dispersion in pyridine - Pacsu and Mullen's method.

Laminarin (5g.) was dissolved in hot water (75ml.) which was then gradually replaced by pyridine. Three 175ml. lots of pyridine were added, taking the volume down to about 75ml. in between by distillation under reduced pressure at 40°. During the last evaporation laminarin commenced to precipitate, the distillation was, therefore, stopped and acetic anhydride (75ml.) added with stirring. The mixture became very hot and was cooled rapidly. When reaction had ceased the solution was left in the dark for 2 days then precipitated into ice-cold water. The fine white acetyl product (II) could not be filtered and was therefore centrifuged off, washed well with water and then dried in a vacuum desiccator (phosphorous pentoxide) to give a glassy solid. Weight: 4.7g. $[\alpha]_D^{18}$ +3.9° (c,1.03 in chloroform). The acetyl content was 29.3%.

III. By direct dispersion in pyridine.

Alcohol-precipitated laminarin (0.75g.) as warmed with pyridine (10ml.) when the polysaccharide swelled completely and formed a gelatinous suspension. To this, acetic anhydride (3ml.) were added dropwise with stirring. Slight warming occured and

the majority of the suspension dissolved. The reaction mixture was allowed to stand for 48 hours in the dark, then poured into water (100ml.). The white precipitate was left in water (3 days), filtered and washed well. Finally, it was precipitated from chloroform solution (20ml.) by the addition of petroleum (b.p. 40-60°) (75ml.),

Weight (dry): 1.26g. i.e. 94% yield.

$[\alpha]_D^{15}$ -59.8° (c,0.96 in chloroform); acetyl content 44.0%.

Reducing power of laminarin acetyl products.

Acetyl products I and III were examined by the same method, namely that of Bergmann and Machemer (18).

About 0.3g. of product were saponified with methanol (25ml.) and approximately 0.1N potassium hydroxide (25ml.) for 4 hours at room temperature. The mixture was exactly neutralised with 2N sulphuric acid (phenolphthalein) and the methanol completely removed by distillation under reduced pressure and the continuous addition of water. 0.1N iodine (5ml.) and 0.2N sodium hydroxide (5ml.) were then added to the aqueous solution of recovered laminarin, and the oxidation completed by standing for 20 minutes at room temperature. After acidification (2ml. 2N sulphuric acid) the liberated iodine was titrated with approximately 0.02N thiosulphate. A blank, omitting the acetyl product, was subjected to the same procedure in order to reduce errors.

Acetyl product I.

0.283g. required 15.9ml. 0.0251N thiosulphate.
Blank " 19.4ml. " "

The consumption of iodine, $4.38.10^{-5}$ moles, for $9.8.10^{-4}$ moles of anhydro-glucose corresponds to the presence of 1 reducing group per 22 residues.

Acetyl product III.

0.3002g. required 20.7ml. 0.0199N thiosulphate.

Blank " 23.7ml. " "

corresponding to the presence of 1 reducing group per 35 residues.

Viscosities of acetyl and methyl laminarin.

The viscosities of several of the acetyl and methyl products were determined in freshly distilled m-cresol using an Ostwald viscometer.

Volume of solution: 10ml. Temperature 20°.

	Time of flow (secs.)	Weight (g.)	η_{sp}	η_{sp}/M'
m-cresol	459			
acetyl I	529	0.1996	0.152	529.10^{-4}
" III	581	0.2002	0.266	925.10^{-4}
methyl I	573	0.2002	0.248	1215.10^{-4}
m-cresol	497			
methyl $IIIa_1$	654	0.2004	0.316	1550.10^{-4}
" $IIIa_2$	649	0.2000	0.306	1500.10^{-4}
" IIIb	633	0.1993	0.294	1440.10^{-4}

M' is the molecular weight of a unit residue i.e. 288 and 204 for the acetyl and methyl derivatives, respectively, corresponding to $C_6H_7O_5(COCH_3)_3$ and $C_6H_7O_5(OCH_3)_3$.

Benzoylation of laminarin.

Experiment showed that alcohol-precipitated and dried laminarin gave highly-coloured solutions with benzoyl chloride and pyridine and poor products resulted, but a satisfactory preparation could be obtained when ordinary laminarin containing some 10% moisture was used. The presence of some water appears to be necessary to prevent interaction of the acylating reagents in this case, since when they were mixed in the absence of polysaccharide, coloured solutions were still obtained.

Laminarin (2g.) was dispersed in pyridine (20ml.) by warming and benzoyl chloride (6ml.) added slowly with vigorous stirring. The mixture became warm, assumed a pale straw-colour and a gummy precipitate was thrown down. After keeping for 12 days in the dark with daily stirring, the whole was poured into water (250ml.) and kept thus for 3 days. The friable buff-coloured powder was filtered, washed free of pyridine and benzoic acid with hot water and then dried in a vacuum desiccator. It was finally dissolved in chloroform (readily soluble) to give a fairly viscous solution which was precipitated with petroleum (b.p. 40-60°) and the product filtered, powdered and dried at 60° in a vacuum overnight.

Weight: 2.66g. $[\alpha]_D^{14}$ -54.9° (c,1.03 in chloroform)

	Found:	C 58.7% ; H 5.23%
laminarin, $C_6H_{10}O_5$,	requires:	C 44.4% ; H 6.18%
mono-benzoyl, $C_{13}H_{14}O_6$,	" :	C 58.7% ; H 5.27%
di-benzoyl, $C_{20}H_{18}O_7$,	" :	C 64.9% ; H 4.87%.

Benzoyl determination.

The material was found to be insoluble in the acetone-potash mixture used for acetyl determination and even on the addition of more acetone to solubilise, the saponification was lengthy. A satisfactory procedure was to dissolve the material (0.1g.) in A.R. acetone (25ml.) and potassium hydroxide solution (10ml.) and to add a further 15ml. of water after several days in order to keep in solution the precipitate which formed. After leaving for a further 2 days the solution and a blank were titrated with 0.1N hydrochloric acid.

0.1060g. of benzoyl product required 4.07ml. 0.1N hydrochloric acid corresponding to 40.4% benzoyl content. A mono-benzoyl compound would contain 39.5% benzoyl.

Methylation of laminarin.

Preparation I.

Laminarin (original sample 9g. dry weight) as stirred into water (50ml.) and 30% sodium hydroxide (100ml.), when the polysaccharide dissolved in a few minutes to give a slightly viscous, straw-coloured solution. Vigorous stirring was

continued at room temperature and 30% sodium hydroxide (100ml.) and dimethyl sulphate (70ml.) added in equal portions at half-hourly intervals over $5\frac{1}{2}$ hours. Considerable frothing occured which was prevented by the addition of capryl alcohol. Stirring was continued overnight.

On careful neutralisation with 50% sulphuric acid, gummy partially methylated laminarin deposited and was removed. The solution was chilled, sodium sulphate removed from it, and then evaporated to dryness under reduced pressure. The resulting solid mixture of inorganic salts and partially methylated laminarin was added to the gum and the whole re-methylated as above in water (50ml.), 30% sodium hydroxide (100ml.) and ethanol (100ml.). After neutralisation the product had lost most of its sticky nature and could be filtered off. The neutral filtrate failed to yield any appreciable amounts of material when extracted with chloroform.

The product was methylated a third time in acetone (100ml.) and 30% sodium hydroxide (100ml.). It was found that the material at this stage could be easily separated from the neutral methylation mixture by boiling, since the partially methylated laminarin was insoluble in hot, but fairly soluble in cold water. The loss of organic solvent also rendered the product more insoluble. The product after separation in this way was washed with hot water until sulphate-free, dissolved

in chloroform which was then dried (anhydrous sodium sulphate), and precipitated with light petroleum (b.p. 40-60°). Methylation was continued until seven treatments had been given; the methoxyl contents from the 4th to 7th stage were 40.5, 42.0, 44.0 and 44.4%, respectively. Calculated for fully methylated laminarin: -OMe 45.6%. Weight of dry purified product: 3.2g. i.e. 28% yield.

$[\alpha]_D^{19}$ -6.5° (c,0.93 in chloroform)

Preparation II.

An attempt was made to improve the yield and to achieve complete methylation.

Fraction A (15g., dry weight) was dissolved in water (100ml.) and 30% sodium hydroxide (400ml.) and methylated at room temperature by the slow addition of dimethyl sulphate (140ml.), as for I. After stirring overnight and partially neutralising with 50% sulphuric acid the solution was evaporated almost to dryness under reduced pressure. The resulting sludge was re-methylated as before with the addition of methanol (250ml.). The mixture after neutralisation, chilling and removal of sodium sulphate, was dialysed for 6 days when it became neutral and sulphate-free. The solution (4L.) was then evaporated to a viscous syrup which was methylated (3rd) in 30% sodium hydroxide (400ml.) and methanol (200ml.) by the addition of dimethyl sulphate (140ml.). Working-up was effected by boiling and

extraction of the filtrate with chloroform. Methylation was continued in acetone until seven treatments had been given, when the methoxyl content was 43.0%. The product (13g.) was then treated twice with Purdie's reagents, namely 100ml. methyl iodide and the gradual addition over 6 hours of 40g. silver oxide. After refluxing overnight the solution was filtered, the silver oxide washed well with hot chloroform and the combined filtrate and washings evaporated to dryness. Purification by precipitation from chloroform yielded a product (10g.), -OMe 43.4%.

This was treated in acetone and 30% sodium hydroxide (250ml.) with dimethyl dulphate (100ml.) but without raising the methoxyl content and since the product gave only 0.5% ash, it was taken to be fully methylated.

Yield: 53%. $[\alpha]_D^{15}$ -6.5° (c,1.0 in chloroform).

Preparation III.

The original sample (21g., dry weight) was methylated at room temperature in 30% sodium hydroxide (550ml.) by the gradual addition of dimethyl sulphate (200ml.) over 5 hours and stirring continued overnight. The partially neutralised reaction mixture was then evaporated to dryness under reduced pressure and methylated as before. Inorganic material was then removed by dialysis (8 days) and the solution (7½L.) evaporated to small

bulk. The product was re-methylated (3rd) by 30% sodium hydroxide (350ml.), acetone (150ml.) and dimethyl sulphate (170ml.) and worked-up by boiling as previously described.

Methylation was continued under these conditions until 9 treatments had been given, purification being effected at the 5th and 7th stages (-OMe 41.7%). The product was then fractionated from chloroform by the addition of petrol giving:

Fraction a: 8.75g. -OMe 41.2%

b: 6.25g. -OMe 43.8% $[\alpha]_D^{13}$ -7.5° (c, 1.02 in chloroform).

The depositing solution was evaporated and the residue re-precipitated from chloroform giving:

Fraction c: 0.75g. -OMe 37.5%.

Total yield at this stage: 60%.

Fraction b (0.5g.) was further methylated twice in boiling methyl iodide (5ml.) by the addition of silver oxide (1.5g.) over 30 hours. On isolation and purification the methoxyl content was found to be unchanged.

Fractions a and c were combined and re-methylated three times by 30% sodium hydroxide (150ml.), dioxan (100ml.) and dimethyl sulphate (75ml.). The product was fractionated as above giving two main fractions:

Fraction a_1: 3.49g. OMe 44.4%

b_2: 3.04g. OMe 43.6%.

Hydrolysis of methylated laminarin I and examination of the products.

Product I (0.1g.) was boiled under reflux with 2% methanolic-hydrogen chloride (2.5ml.) for 32 hours and the solution neutralised with silver carbonate, filtered and evaporated under reduced pressure to a syrup. The methyl glucosides were then hydrolysed with N sulphuric acid (2.5ml.) for 4 hours on a boiling-water bath and then solution neutralised with barium carbonate.

The neutral hydrolysate, and authentic specimens of 2:3:6-trimethyl glucose, 2:4:6-trimethyl glucose and 2:3:4:6-tetramethyl glucose were examined on a paper chromatogram in the usual way (15 hours running time, butanol solvent.). On development with aniline oxalate pink spots having the same R_G values as the last two sugars, developed for the hydrolysate, and also a third spot, R_G value about 0.55, that is in the "dimethyl hexose" region. Traces of monomethyl sugars and possibly glucose could also be detected.

Methanolysis of methylated laminarin II.

Product II (7.38g.) was boiled under reflux with 2% dry methanolic-hydrogen chloride (200ml.) and since solution did not occur after 2 hours heating, chloroform (35ml.) was added. After a constant rotation was reached (50 hours), the solution

was cooled, neutralised to Congo Red with silver carbonate, filtered, washing the residue well with chloroform, and evaporated to an amber-coloured syrup II, n_D^{17} 1.4597. Weight: 8.16g. Syrup II did not reduce Fehling's solution and hydrolysis and paper chromatography confirmed the above observations.

Hydrolysis of Syrup II and separation on a column of cellulose.

Syrup II (3.210g.) was hydrolysed with 2N hydrochloric acid (100ml.) for 4 hours in a boiling-water bath. The hydrolysate was cooled, neutralised with silver carbonate and filtered hot, washing the residue well with hot water. Excess silver was removed from the filtrate with hydrogen sulphide and the solution evaporated to 50ml. when 2ml. was removed for paper chromatographic analysis. The remainder was taken to dryness, and the white crystals thoroughly dried with alcohol-benzene. Weight: 2.715g.

In this and in all other chromatographic separations the columns were prepared with Whatman ashless filter tablets powdered in a hammer mill to be able to pass an 80-mesh sieve. They were packed to manual tightness and pre-washed with water, butanol and then the solvent. Fractions (3-5ml.) of the eluate were removed in test tubes by a type of fraction cutter described by McGilvray (22) and the contents of every 10th or 20th examined by paper chromatography. After quantitatively collecting together suitable fractions the solvent was removed under reduced pressure

at as low a temperature as possible. The residues were taken up into water, treated with charcoal (hot) and the solutions after filtering through a minimum of "FilterCel", were evaporated to dryness. Final drying was normally effected in a vacuum at 35° overnight.

The sugar mixture (2.715g.) dissolved in the chromatogram solvent (40ml. of 60% purified petroleum (b.p. 100-120°)/40% butanol (by volume), saturated with water), was introduced on to the column (3.5 x 41cm.) and elution commenced. 200ml. was collected before fractions (250) were taken. On examination:

Tubes 30-70 contained tetramethyl glucose.

" 72,74 & 76 " no sugar.

" 80-230 " trimethyl glucose.

" 240-250 " no sugar.

Therefore tubes 25-75 were collected giving 268mg. of syrup which partially crystallised.

Tubes 76-230 yielded 2.190g. of white chromatographically pure crystals, m.p. 115-7°. This weight of trimethyl glucose represents 80.7% of the starting material.

The column was finally eluted with water but this fraction was accidentally lost during working up:

Examination of "tetramethyl" fraction.

This gave the following analyses:

-OMe 49.2% (tetramethyl hexose requires -OMe 52.6%)

$[\alpha]_D^{13}$ +68.7° (c,1.0 in water) (2:3:4:6-tetramethyl glucose has $[\alpha]_D$ +83.3°).

4.98mg. and 5.22mg. consumed 2.55ml. and 2.65ml. of 0.01083N iodine, respectively, when oxidised in buffered hypoiodite solution (phosphate buffer, pH 11.4, see ref. 46, 21). These figures correspond to the presence of 52.0 and 51.5% tetramethyl glucose.

It was therefore evident that the fraction was very impure and the high methoxyl content indicated that some trimethyl-methyl-glucoside may be contaminating the syrup. This was confirmed by hydrolysis and examination by paper chromatography when the presence of trimethyl glucose was revealed. Accordingly, the remainder of the fraction (178mg.) was hydrolysed for 2 hours with 2N hydrochloric acid (10ml.) at boiling-water temperature and the hydrolysate worked up to give 150mg. of a completely crystalline mixture of tetramethyl glucose and trimethyl glucose.

The mixture (150mg.) was then separated on a small cellulose column (1.6 x 37cm.) to give tetramethyl glucose (102mg.) and trimethyl glucose (28mg.). Recovery: 90%. Hydrolysis of this "tetramethyl" fraction furnished no trimethyl glucose but analysis gave:

-OMe 47.5% (corresponding to 90% tetramethyl glucose)

$[\alpha]_D$ +67.5° (c,0.96 in water) (corresponding to 81% tetramethyl glucose)

3.96mg. consumed 2.32ml. 0.0096N iodine, corresponding to 70% tetramethyl hexose.

The individual analyses still conflict and the true amount

of tetramethyl glucose was ascertained by crystallisation from petroleum (b.p. 40-60°), when 55mg. of pure sugar (-OMe 50.4%, $[\alpha]_D$ +83.3°) were obtained from 82mg. of impure material, representing 70% recovery. It was generally found that the methoxyl content of fractions isolated from cellulose columns tended to be high even though the sugar content was sometimes low.

Assuming a pure sugar content of 70% in the final analysis, the total weight of end-group, by proportion, becomes 127mg. If the proportion of dimethyl sugars is assumed to be 8.5% of the total (subsequent work), the total recovery is about 2.56g. (94%) amd the proportion of end-group becomes about 4,6%, corresponding to a repeating unit of 21.7 residues. No great reliance can be placed in this figure, however, in view of the large number of steps and analyses in the assessment.

Authentication of end-group.

The end-group after recrystallisation from petroleum (b.p. 40-60°) had m.p. 83-85°, not depressed on admixture with 2:3:4:6-tetramethyl glucose. $[\alpha]_D^{15}$ +83.3° (c,0.87 in water, 12 hours); pure 2:3:4:6-tetramethyl-D-glucose had: $[\alpha]_D^{15}$ +83.4° (c,1.78 in water). Analysis:

Found: C 51.3% ; H 8.53% ; -OMe 50.4%

Calculated for $C_{10}H_{20}O_6$: C 50.9% ; H 8.48% ; -OMe 52.6%.

The anilide was prepared by boiling the sugar (75mg.) under reflux with redistilled aniline (30mg.) in absolute ethanol (3ml.) for 2 hours. The solvent was removed in a disiccator and the residue recrystallised three times from dry ethyl acetate to give colourless needles m.p. 134-6°, m.m.p. 135-6° with authentic 2:3:4:6-tetramethyl glucose anilide.

Found: C 61.3% ; H 8.01% ; N 3.65% ; -OMe 39.2%.

Calculated for $C_{16}H_{25}O_5N$: C 61.8% ; H 8.04% ; N 4.50% ; -OMe 39.9%.

The low N content is probably analytical error.

Authentication of the trimethyl glucose.

On recrystallisation from dry ether the m.p. was raised from 115-7° to 124-6°.

$[\alpha]_D^{16}$ +91.3° (initial) → +75.5° (12 hours, equilibrium, c, 2.2 in water).

Found: C 49.3% ; H 8.26% ; -OMe 41.3%

Calculated for $C_9H_{18}O_6$: C 49.1% ; H 8.12% ; -OMe 41.8%.

Preparation of 2:4:6-trimethyl gluconolactone.

The sugar (0.7g.) was oxidised with bromine (1ml.) in water (3ml.) until non-reducing (48 hours). Bromine was removed by aeration and the solution neutralised with silver carbonate. After filtration and washing the residue, excess silver was removed by hydrogen sulphide. Silver sulphide was removed and the solution evaporated to dryness and the residue heated in a vacuum at about 80° for 3 hours to complete the lactone

formation. It was then extracted with warm dry ether which was removed and the lactone distilled at 145°/0.01mm. to give a colourless syrup. n_D^{16} 1.4682. Weight: 0.56g.
$[\alpha]_D^{17}$ +95° (initial)⟶+42.7° (6 hours, constant, c,2.2 in water).

Amide formation.

The lactone (0.2g.) was allowed to stand with methanolic ammonia (10ml.) for 5 days at room temperature. Solvent was removed in a vacuum desiccator to give a syrup which could not be crystallised from acetone-petroleum (three authors had previously crystallised this amide, m.p. 99-100°). The syrup was clarified with charcoal in acetone, and then had $[\alpha]_D^{16}$ +35.0°(c,1.1 in acetone).

Attempted phenylhydrazide formation.

The lactone (0.1g.) and freshly distilled phenylhydrazine (0.1g.) were refluxed together in dry ether (3ml.) for 1½ hours.; on removing the solvent a yellow oil remained which was titurated with light petroleum to remove excess phenylhydrazine. The resulting viscous syrup could not be crystallised from ether-petroleum or on leaving at 0° for some months.

Preparation of 2:4:6-trimethyl glucose anilide.

The sugar (0.25g.) and freshly distilled aniline (0.1g.) were boiled in absolute ethanol (12.5ml.) for 2 hours and the solvent then removed in a vacuum desiccator. On re-crystallisation

of the residue from dry ethyl acetate (3 times), white crystals m.p. 163-5° were obtained. Yield: 70mg.

$[\alpha]_D^{15}$ -81.0° (c,0.58 in dry methanol) 20 hours, constant.

Found: C 59.9% ; H 7.62% ; N 4.91%.

Calculated for $C_{14}H_{23}O_5N$: C 60.2% ; H 7.68% ; N 4.68%.

Methanolysis of methylated laminarin III.

Fractions $IIIa_1$, $IIIa_2$ (2.5g. each) and fraction IIIb (4g.) were boiled together under reflux in 2% methanolic hydrogen chloride for 54 hours (constant rotation). Working up in the usual manner gave a clear, golden syrup III, n_D^{16} 1.4608, which did not reduce Fehling's solution. Weight, 9.69g.

Hydrolysis of syrup III and separation of a column of cellulose.

1st Experiment.

The syrup of methyl glucosides III (4.19g.) was hydrolysed to constant rotation (7 hours) by 2N hydrochloric acid (120ml.) at boiling water temperature, and the hydrolysate worked up after neutralising with silver carbonate in the usual manner. Some of the neutral solution was removed for paper chromatography experiments (see p.98) and the remainder taken to dryness to give 2.98g. of crystals, alcohol-benzene dried.

The sugar mixture (2.98g.) was then separated on a column of cellulose (3.5 x 41cm.) as described above, and gave:

309mg. "tetramethyl" fraction.

2259mg. pure 2:4:6-trimethyl glucose.

194mg. "dimethyl" and "monomethyl" fraction (eluted together with water).

Total recovery: 2762mg. i.e. 93%.

Examination of the "tetramethyl" fraction.

The partially crystalline fraction (309mg.) was hydrolysed with 2N hydrochloric acid (10ml.) for 4 hours at boiling water temperature and the hydrolysate worked up to give 291mg. of a mixture of tetramethyl and trimethyl glucose. This was then separated on a cellulose column (3.5 x 41cm.) to give:

160mg. crystalline chromatographically pure tetramethyl glucose.
100mg. " " " trimethyl " (eluted with water).

Recovery: 89%. Analysis of the end-group gave:

-OMe 49.8% (corresponding to 95% tetramethyl glucose)

$[\alpha]_D$ +65.8° (c,1.26 in water) " " 79% " "

5.18mg. consumed 3.56 and 3.68ml. 0.0098N iodine, corresponding to 79.7 and 82.2% tetramethyl glucose.

Thus the actual weight of tetramethyl glucose isolated is 80% of 160mg. i.e. 128mg. Accounting for the recovery from the final separation (89%), 144mg. tetramethyl glucose has been obtained from a total of 2762mg. sugars, representing 4.9% end-group.

2nd Experiment.

Syrup III (3.56g.) was hydrolysed with 2N hydrochloric acid (100ml.) for 11 hours at boiling-water temperature and the hydrolysate worked up to give 3.213g. of crystals, dried in vacuo for 8 hours at 35°. The sugars were then completely separated on a cellulose column (3.5 x 41cm.) to give:

250mg. "tetramethyl" fraction (eluted with 70% petroleum/30% butanol).
2481mg. pure 2:4:6-trimethyl glucose (eluted with 50% petroleum/50% butanol).
220mg. "dimethyl" fraction " " " " "
47mg. "monomethyl" and glucose fraction (eluted with water).

Total recovery: 2998mg. i.e. 93%.

Examination of the "tetramethyl" fraction.

The fraction (250mg.) was hydrolysed with 2N hydrochloric acid (10ml.) for 4 hours at boiling-water temperature and the mixture of sugars isolated in the usual manner to give 230mg. crystals, dried in a vacuum for 4 hours at 35°. On separation this gave:

148mg. crystalline tetramethyl glucose.
38mg. " trimethyl " (eluted with water).

Recovery: 81%. Analysis of the end-group gave:

-OMe 49.0% (corresponding to 93% tetramethyl glucose).
$[\alpha]_D^{15}$ +77.0° (c,1.0 in water) " " 93% " "

Thus the actual weight of end-group is 93% of 148mg. i.e. 138mg. or accounting for the recovery (81%), 170mg. has been isolated from 2998mg. total, representing 5.3% end-group.

Examination of the "dimethyl" fraction.

The fraction was spread over about 600 tubes but no difference between the R_G values of the first and last fractional cuts could be detected. However, it is very probable that it is composed of two dimethyl sugars, 2:6- and 4:6-dimethyl glucoses, and this aspect will be examined below in the section on demethylation. It was isolated as a glass which slowly partially crystallised; -OMe 27.7% (a dimethyl hexose requires -OMe 29.8%) and mutarotated slightly: $[\alpha]_D^{16}$ +77.2° (20 mins.)→+68.6° (260 mins., constant, c,1.4 in water). 2:6-dimethyl glucose (a glass) has $[\alpha]_D$ +58.3° (in water) or 63.3° (in water) (26) and 4:6-dimethyl glucose has $[\alpha]_D^{18}$ +108° (3 mins.)→+65.7° (1140 mins., in water) (47). It is therefore almost pure dimethyl hexose and the weight (220mg.) corresponds to 7.8% dimethyl glucose in the mixture of 2998mg. total.

Quantitative paper chromatographical analysis of Syrup III.

It was found that the analytical method of Hirst, Hough and Jones (21) gave very high end-group analyses when applied to a 4 hour hydrolysate of syrup II and consistent and reasonable results were only obtained when the time of hydrolysis had been lengthened to 7 hours and the hydrolysate before chromatography had been rendered completely free of inorganic ions. The reason for this probably lies in incomplete hydrolysis

and extraction of trimethyl methyl glucoside together with the tetramethyl glucose from the chromatogram. Hydrolysis of the methyl glucoside then led to apparently high reducing sugar content in the end-group extract.

The procedure adopted was to neutralise carefully a 7-hour hydrolysate (see 1st Experiment above), remove the silver and then pass through small columns of prepared "Zeo-Karb HI" and "De-acidite" in turn. The rigorously ion-free solution was evaporated to a thin syrup and then analysed for tetramethyl, trimethyl and dimethyl glucoses as described in the above paper. No control sugar was added since it was assumed that these three sugars made up almost the whole of the hydrolysate; a simple proportionality of the molar consumptions of iodine then directly gives the composition of the mixture. The more convenient phosphate buffer (pH 11.4) replaced the bicarbonate buffer used in the original method.

The following results were obtained:

Titres (ml. 0.01N thiosulphate)			% Tetramethyl / Trimethyl (by wt.)	% Dimethyl / Trimethyl (by wt.)
Tetramethyl glucose	Trimethyl glucose	Dimethyl glucose		
	5.48	0.50		9.0
0.51	7.54	0.75	7.2	9.8
0.50	7.68		6.9	
	9.92	0.87		8.6
0.58	9.36	0.95	6.4	9.5
		Average:	6.8±0.4%	9.3±0.6%

Whence: $\frac{\text{number of moles of tetramethyl glucose}}{\text{" " " " total sugars}} = \underline{5.4} \pm 0.3\%.$

$\frac{\text{number of moles of dimethyl glucose}}{\text{" " " " total sugars}} = \underline{8.3} \pm 0.5\%.$

"Demethylation" of 2:4:6-trimethyl glucose.

The conditions of methanolysis and hydrolysis of methylated laminarin were simulated as closely as possible.

7-hour treatment with 2N hydrochloric acid.

Pure crystalline 2:4:6-trimethyl glucose (0.2g.) was boiled under reflux with 2% methanolic hydrogen chloride (7ml.) and chloroform (1.3ml.) for 54 hours. The solution was neutralised with silver carbonate, silver was removed with hydrogen sulphide and a syrup of methyl glucoside obtained by evaporation. The syrup was then hydrolysed with 2N hydrochloric acid.(6ml.) for 7 hours at boiling-water temperature and the sugars isolated in a thin syrupy, ion-free form. Paper chromatographical analysis then gave the following results:

Titres (ml.0.01N thiosulphate)		% Dimethyl / % Trimethyl	% moles dimethyl / % " dimethyl and trimethyl
Trimethyl glucose	Dimethyl glucose		
9.19	0.44	4.5	4.36
6.28	0.31	4.6	4.47

11-hour treatment with 2N hydrochloric acid.

Pure 2:4:6-trimethyl glucose(2.6g.) was boiled in 2% methanolic hydrogen chloride (84ml.) and chloroform (17ml.) for 52 hours and the resulting syrup of methyl glucosides

hydrolysed with 2N hydrochloric acid (75ml.) for 11 hours at boiling water temperature. After working up in the usual manner, the white crystalline mixture of sugars weighed 2.596g., dried for 7 hours in vacuo at 35°. Examination on the paper chromatogram showed considerable amounts of dimethyl glucose and traces of monomethyl glucoses.

The mixture was separated on a cellulose column (3.5 x 40cm.) to give:

2164mg. 2:4:6-trimethyl glucose (eluted with 50% petroleum/50% butanol).
202mg. "dimethyl" fraction " " " " "
119mg. monomethyl glucoses, glucose and inorganic material (eluted with water).

Recovery: 2485mg. i.e. 95.7%.

The weight of dimethyl fraction represents 8.7% of the total recovered sugars, when expressed in numbers of moles. It was a glass which slowly crystallised and had $[\alpha]_D^{16}$ +67.8° (c,1.4 in water), with hardly any mutarotation.

Examination of the "dimethyl" fractions from methylated laminarin and "demethylation".

Because of the similarity of these two fractions they are discussed together.

Both gave only one spot (R_G value about 0.55) on the paper chromatogram in butanol/ethanol/water even when the length of run down the paper was over 30cm. The spot when developed with

aniline oxalate, however, appeared to be composite, having a pink body and a brown "tail" towards the slower portion of the oval. 2:6- and 4:6-dimethyl glucoses gave spots of identical R_G values as each other and as the dimethyl fractions, whilst 2:4-dimethyl glucose had a higher R_G value than these. Also, 2:6- and 4:6-dimethyl glucoses gave pink and brown colours, respectively, with aniline oxalate.

A portion of the dimethyl fraction from methylated laminarin on crystallisation from dry ethyl acetate gave clusters of small needles m.p. 152-6° which on re-crystallisation gave fine needles m.p. 159-62°, m.m.p. 155-9° with authentic 4:6-dimethyl glucose (m.p. 154-9°).

The production of formaldehyde on periodate oxidation was tested for as follows (25):

The dimethyl fraction (13mg.) was placed in phosphate buffer, pH 7.4 (10ml.), 0.25M sodium periodate (2.4ml.) added, and the solution left for $2\frac{1}{2}$ hours at room temperature. 2N hydrochloric acid (1.5ml.) was then added and the liberated iodine removed by 0.4N sodium arsenite (12ml.). The solution was buffered at pH 4.5 by a 0.2N acetate buffer (16ml.) and dimedone (160mg.) in ethanol (2ml.) added. After heating on a boiling water bath for 2 hours the mixture was kept at room temperature but no precipitate formed even after leaving overnight. A control oxidation with 2:4-dimethyl galactose anilide gave a definite crystalline

precipitate after initial difficulty through liberation of aniline. Glucose gave a bulky crystalline precipitate m.p. 189-90°.

Periodate oxidation of 4:6-dimethyl glucose and dimethyl fractions.

(a) 4:6-dimethyl glucose.

The sugar (38.4mg.), containing a trace of monomethyl sugar which could not be removed even after 4 re-crystallisations, was made up in aqueous 0.5M sodium periodate (1.036g.) and the optical rotation observed at intervals in a ½dm. micro-tube.

Rotation (deg.)	+0.64	+0.58	+0.54	+0.49	+0.49	+0.44	+0.44
Time (hrs:mins.)	0:15	0:45	1:30	2:30	19:00	48:00	96:00

For the derived 2:4-dimethyl-D-erythrose:

$[\alpha]_D^{13}$ +33.4° (c, 2.64 in 0.5M sodium periodate).

(b) Dimethyl fraction from methylated laminarin.

This fraction (43.2mg.) in aqueous 0.5M sodium periodate (1.031g.) after filtering off a trace of insoluble matter, was examined in a ½dm. micro-tube:

Rotation (deg.)	+0.86	+0.84	+0.32	+0.27	+0.24	+0.26	+0.26	+0.24
Time (hrs:mins.)	0:25	1:00	23:00	26:00	28:00	29:30	30:30	47:00

Taking the final rotation of this 4.18% solution to be +0.24° by comparison with that for 4:6-dimethyl glucose (+0.44, 3.71%), the percentage of the latter in the fraction becomes:

$$\frac{0.24}{0.44} \cdot \frac{3.71}{4.18} \cdot 100, \text{ i.e. } 48.5\%.$$

(c) Dimethyl fraction from "demethylation".

As for (b), 41.6mg. fraction in 1.04g. periodate solution:

Rotation (deg.)	+0.79	+0.77	+0.74	+0.55	+0.39	+0.26	+0.26	+0.26
Time (hrs:mins.)	0:20	1:30	2:00	19:00	43:00	67:00	91:00	113:00

Whence the percentage of 4:6-dimethyl glucose is:

$$\frac{0.26}{0.44} \cdot \frac{3.71}{3.99} \cdot 100, \text{ i.e. } \underline{53.5\%}$$

N.B. Sufficient 2:6-dimethyl glucose was not available for the desirable check on its behaviour with periodate.

Periodate oxidation of laminarin.

Estimation of formic acid.

(a) Oxidation in diffuse daylight.

In the first experiments, estimates were started after 5 days oxidation but it was found necessary to examine the course of oxidation at 1 and 3 days in order to obtain a complete picture of the reaction. Slightly different weights were used in these two series of experiments but in all cases the titres are corrected to accommodate these differences. The following procedure was adopted for the original sample, fraction A and fraction B.

The sample (ca. 1g.) was weighed into a dry 250ml. stoppered bottle, potassium chloride (2g.) and 0.25M sodium metaperiodate (10ml.) were added and the volume made up exactly to 100ml. with distilled water. A blank omitting the polysaccharide was also made up. The bottles were tightly stoppered and shaken continuously for 5 days in diffused daylight and thereafter

allowed to stand in the dark. In most cases the polysaccharide would not settle out of solution rapidly enough to permit the extraction of aliquots at intervals and therefore the suspension (ca. 35ml.) was centrifuged and exactly 20ml. of the clear supernatant solution pipetted for titration. The remaining solution and solid were returned without dilution to the reaction bottle. In this way a check was kept on the concentration of the formic acid. Excess periodate in the 20ml. was destroyed by the addition of ethylene glycol (0.5ml.), and after allowing to stand for 5 minutes to complete this process, the formic acid was titrated with approximately 0.01N sodium hydroxide (methyl red). The following volumes (ml.) of 0.01N sodium hydroxide were required to neutralise 20ml. of solution:

Days	1	3	new experiment → 5	6	8	11
Blank	0.03					
Original	12.15	15.33	16.16	16.51	17.60	20.04
Fraction A	9.88	12.39	13.12	13.39	14.69	17.84
" B	14.33	18.08	18.70	19.13	19.52	19.99

The titre for the original sample is corrected for slight initial acidity.

Dry weights: Original sample: 0.902g.

Fraction A: 0.763g.

" B: 0.847g.

A slight liberation of iodine (faint fawn colour) was noticed after about 10 days in all cases.

The titres for extractions after the first are not true since the volume is being progressively reduced although the same weight of insoluble polysaccharide remains in the system. This means that the formic acid liberated after the first extraction is entering a smaller volume than originally contained all the laminarin. Hence the titres for 3, 6, 8 and 11 days are too high.

Suppose the titres for 5, 6, 8 and 11 days (new experiment) are a, x, y and z, respectively. The first titre, a, is correct. The additional formic acid liberated between 5 and 6 days, (x-a), has entered a volume of 80ml. instead of 100ml., thus corrected to 100ml. the true additional amount is $\frac{4}{5}$ (x-a) and therefore the true titre at 6 days is: $a + \frac{4}{5}(x-a)$. Similarly for the 8th day the true titre is: $a + \frac{4}{5}(x-a) + \frac{3}{5}(y-x)$, etc.

Corrected in this way, and for blanks, the true titres are:

Days	1	3	5	6	8	11
Original	12.12	14.78	16.13	16.42	17.07	18.05
Fraction A	9.85	11.92	13.09	13.31	14.09	15.33
" B	14.30	17.32	18.67	19.05	19.28	19.28

These results are shown in Graph III (p.50), but they have

been halved for convenience in comparing with the curve for oxidation in the dark, in which 10ml. extracts were titrated.

Original. Titre at 4 days (graph III): 7.75ml.

In all the titrations of formic acid, the titre has to be corrected by +3% since the pK value of methyl red is about 5. With this correction the liberation of formic acid per 100ml. solution is $79.8.10^{-5}$ moles. The weight of polysaccharide is 0.902g. i.e. $5.57.10^{-3}$ moles of anhydro-glucose, thus the liberation of formic acid is:

1 mole per 7.0 moles anhydro-glucose

At 5 days the figure is 6.7 moles.

Fraction A. Titres at 4 and 5 days: 6.24 and 6.50ml.

Weight of polysaccharide: 0.763g. i.e. $4.71.10^{-3}$ moles anhydro-glucose, whence the corresponding figures are 7.3 and 7.0, respectively.

Fraction B. Titres at 7 days: 9.63ml.

Weight: 0.847g., i.e. $5.23.10^{-3}$ moles of anhydro-glucose giving a figure of 5.3.

(b) Oxidation in the dark:

The original sample (ca.1g.) was shaken with potassium metaperiodate (0.5g.), potassium chloride (2g.) and distilled water (100ml.) in a 250ml. stoppered bottle from which light was completely excluded by means of a dark cloth wrapping. A blank was similarly made up. At intervals the suspension (15ml.) was

centrifuged and 10ml. extracted for titration with 0.01N sodium hydroxide as above. Shaking was stoppered after 8 days. The following actual and corrected titres were obtained:

Days	1	3	5	6	8	11	15
Actual titre (ml. 0.01N sodium hydroxide)	7.04	8.40	9.26	9.72	9.95	10.68	11.33
Corrected titre (ml. 0.01N sodium hydroxide)	7.04	8.26	8.95	9.27	9.40	9.77	10.13

These results are also shown on Graph III (p.50).

Traces of iodine were observed after 14 days.

From Graph III: Titres at 4 and 5 days are:

8.62 and 8.95ml., respectively.

Weight of polysaccharide: 1.014g. i.e. $6.26.10^{-3}$ moles anhydro-glucose, whence the liberation of formic acid corresponds to 1 mole per 7.1 and 6.8 moles anhydro-glucose, respectively.

(c) Oxidation of Dr. V. C. Barry's sample:

The procedure was identical to that of (a), the reaction being carried out in the light.

Dry weight: 0.4610g.

Days	4	5	7
Actual titre (ml. 0.01N per 20ml. solution sodium hydroxide)	14.34	14.52	15.00
Corrected titre (ml. 0.01N per 20ml. solution sodium hydroxide)	14.34	14.48	14.77

An appreciable correction (1.81ml.) for acidity of the sample had to be made. There is a small steady increase in acid

content and at 4 days the titre (14.34ml.) corresponds to the liberation of 1 mole of formic acid per 3.9 anhydro-glucose residues.

Consumption of periodate.

In an attempt to determine the consumption of periodate at intervals during the reaction, laminarin was oxidised with the soluble sodium metaperiodate, but oxidation was so rapid and the symptoms of over-oxidation so pronounced that this was abandoned. Instead the total consumption of periodate was determined at 5 days using potassium periodate in the normal way.

(a) Oxidation in diffuse daylight.

Fraction A (0.1725g.) was weighed into a 2 onz. stoppered bottle and 0.25M sodium metaperiodate (5ml.), potassium chloride (1g.) and distilled water (5ml.) added. The bottle, together with a blank omitting the polysaccharide, was shaken in diffuse daylight for 5 days and the total contents estimated for periodate. That is, solid sodium bicarbonate (2g.) and potassium iodide (1g.) were added and the liberated iodine titrated with 0.1N sodium arsenite.

The laminarin and blank required 18.8 and 24.2ml. 0.01N arsenite, respectively, whence the consumption of periodate ($2IO_4^- \equiv As_2O_3$) is 1 mole per 3.9 moles of anhydro-glucose.

(b) Oxidation in the dark:

The original sample (0.1960g.) was shaken for 5 days in

a completely darkened bottle (2 onz.) with potassium metaperiodate (0.2g.), potassium chloride (1g.) and distilled water (10ml.). A blank was made up with exactly the same weight of periodate.

The titres were: 17.43 and 10.70ml. 0.1008N sodium arsenite, corresponding to a consumption of 1 mole of periodate per 3.6 moles of anhydro-glucose. This figure may be a little high since some iodine was liberated in the oxidation.

Estimation of formaldehyde during periodate oxidation of laminarin.

That formaldehyde is produced during oxidation was demonstrated by a colour test as follows:

Laminarin was oxidised as above and the suspension centrifuged. The clear supernatant liquid was acidified and periodate and iodate reduced by sodium arsenite. To this solution was added an equal volume of a dilute solution of potassium ferricyanide and recrystallised phenylhydrazine hydrochloride followed by 2 drops of concentrated hydrochloric acid. The port wine colour diagnostic of formaldehyde was produced in a few seconds.

The original sample (2.294g.) was shaken for 4 days in daylight in a stoppered bottle with potassium metaperiodate (1.2g.) and distilled water (20ml.). The suspension, which showed the presence of iodine, was then centrifuged and formaldehyde determined in 5ml. of the clear solution by precipitation as the formaldehyde-dimedone complex:

The solution (5ml.) was acidified with 2N hydrochloric acid (1ml.) and 0.4N sodium arsenite (10ml.) added. Much iodine was precipitated which was filtered off, and the filtrate and washings collected quantitatively. Further arsenite (5ml.) was then required to remove the remaining iodine. The solution was buffered at pH 4.5 (10ml. of 0.2N sodium acetate - acetic acid), dimedone (160mg.) in ethanol (2ml.) added and the solution heated for 20 minutes on a boiling water bath and then left at room temperature overnight. The crystalline precipitate of formaldehyde-dimedone was filtered through a sintered weighing stick, washed with water (50ml.) and dried for 1 hour in a vacuum at 60° over phosphorous pentoxide.

Weight of F.D. found: 35.95mg., 35.80mg. ; m.p. 189-90°.

The recovery of formaldehyde under the above conditions of oxidation and analysis is not quite quantitative (93%) (see below), whence the mean weight of formaldehyde found becomes: $\frac{35.87}{0.93}$. 0.1027mg. from 0.573g. laminarin.

(0.1027g. formaldehyde are contained in 1g. F.D.)

This corresponds to the liberation of 1 mole of formaldehyde per 26.7 anhydro-glucose residues.

Estimation of formaldehyde under the same conditions.

An approximately 0.4% formaldehyde solution (3ml.) was shaken for 4 days with distilled water (17ml.) and potassium

periodate (1.2g.). After this time the solid was allowed to settle and the formaldehyde was estimated in 5ml. portions as follows:

2N hydrochloric acid (1ml.) was added followed by 0.4N arsenite (15ml.), filtration of iodine being unnecessary in this case. Acetate buffer (10ml.) and dimedone reagent (2ml.) were added and the solution heated for 15 minutes and cooled overnight.

Weight of F.D. found: 25.69mg., 25.59mg.

The same 0.4% solution (3ml.) was diluted with water (17ml.) and formaldehyde estimated in 5ml. portions in the same way, omitting acidification and treatment with arsenite.

Weight of F.D. found: 27.59mg., 27.67mg.

Whence the recovery of formaldehyde is $\frac{25.64}{27.63} \cdot 100$, i.e. 93%.

Periodate oxidation of laminaribiose.

Laminaribiose (47.7mg., $1.4.10^{-4}$ moles) in distilled water (25ml.) was oxidised by shaking in daylight with potassium metaperiodate (0.3g.) and potassium chloride (0.6g.) (pH of solution: 8). A blank was also made up omitting the sugar. At intervals, portions (5ml.) were extracted, ethylene glycol (0.3ml.) added and the whole titrated with 0.01N sodium hydroxide (methyl red) after allowing to stand (5 mins.). The following titres (with

+3% indicator correction) were obtained:

Days	2	4	6	9
Titre (ml. 0.01N sodium hydroxide)	4.28	6.57	7.97	9.27
Moles formic acid per mole disaccharide	1.53	2.35	2.85	3.31

Iodine was observed after the 5th day. The results have been plotted on Graph IV (p.53).

Molecular weight determination by Barger's method.

Barger's method consists essentially in finding the condition of equal vapour pressure of two solutions in the same solvent, the molarity of one and the concentration of the other being known. Knowing the molecular weight of the solute in the former solution, that of the latter can immediately be deduced, since solutions containing the same number of molecules in the same solvent depress the vapour pressure an equal amount. This condition is found by enclosing the solutions as drops in capillaries and measuring the variation in size of these drops. Originally the technique was confined to low molecular weight compounds but it has been found possible to examine polymers of M.W. 1000-100,000 by this method (42).

In the present instance the size of the drops was measured by a travelling microscope having a vernier accurate to 0.02mm., the capillaries being kept under water in a constant temperature

room. The accuracy of measurement was not as high as is ideally desired, but sufficed for an approximate result. Chloroform solutions of known molarity were made up from sucrose octaacetate and compared with a 1.05% solution of methylated laminarin (fraction $IIIa_2$). After making up the capillaries, they were allowed to come to a steady temperature for 1 hour and then the first measurements taken. Final observations were taken after several hours. The result of two experiments are given:

	Molarity of sucrose octa-acetate drops	Change in size of drops (10^{-2} mm.)					Sum of changes with respect to methylated laminarin
		L	S	L	S	L	
3 hrs.	5.10^{-3}	0	+6	0	+2	-4	-12
	4. "	+2	+2	+4	+2	-2	0
	3. "	+4	+4	+4	+4	0	0
	2. "	+8	-2	+4	-2	+2	+18

L and S represent drops of methylated laminarin and sucrose octaacetate, respectively.

5 hrs.	5.10^{-3}	+2	+6	-2	+4	-4	-16
	4. "	0	+6	+2	+4	-2	-10
	3. "	+4	+6	+6	+4	+4	+6
	2. "	+10	-6	+8	0	+4	+28
18 hrs.	5.10^{-3}	+2	+8	+2	+8	+2	-10
	4. "	+4	+8	+2	+10	-2	-12
	3. "	+14	+6	+10	+4	+4	+18
	2. "	+32	-14	+18	+10	+8	+62

It appears that the isopiestic condition lies between 3 and 4.10^{-3} molar, whence the M.W. of methylated laminarin is:

$$\frac{1.05}{3.10^{-3}} \cdot 10 \text{ to } \frac{1.05}{4.10^{-3}} \cdot 10 \text{ i.e. } \underline{3,500\text{-}2,600}.$$

Since the molecular weight of a unit residue is 204, the number of residues is: 13 to 17.

Experiments with acetylated laminarin gave inconsistent results, all the drops tending to increase in size - a phenomenon also noted by Barger when the concentration of the solution was small.

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SUMMARY

1. Laminarin, a polysaccharide from L. cloustoni, has been shown to be composed wholly of D-glucose.

2. The polysaccharide has been partially hydrolysed and pure samples of the disaccharide building unit, laminaribiose, isolated. The properties of the disaccharide have been examined, in particular its behaviour towards periodate and dinitrosalicylic acid in alkaline solution. Laminaribiosazone has been identified with that obtained by other workers.

3. Acetolysis of laminarin failed to yield any useful crystalline derivatives.

4. The reducing power of laminarin corresponded to a molecular size of 40-45 residues, whilst reaction with 3:5-dinitrosalicylic acid gave a much lower figure, probably through degradation.

5. Triacetyl, monobenzoyl and methylated laminarin (-OMe 44%) have been prepared.

6. Methanolysis and hydrolysis of methylated laminarin gave a mixture of sugars which was analysed by paper chromatography and partition chromatography on cellulose. 5% end-group, authenticated as 2:3:4:6-tetramethyl glucose by isolation of the crystalline sugar and anilide, and 8% dimethyl glucoses have been obtained, the latter probably an equimolecular mixture of 2:6- and 4:6-dimethyl glucoses. The bulk of the

mixture (85%) was authenticated as 2:4:6-trimethyl glucose by isolation of the crystalline sugar, anilide, lactone and amide.

7. Demethylation of pure 2:4:6-trimethyl glucose furnished about 4% and 8% dimethyl glucoses after 7 and 11 hours treatment with 2N hydrochloric acid at 95-100°, respectively. The mixture of dimethyl glucose was similar to that from methylated laminarin.

8. Periodate oxidation of laminarin has been examined in some detail. Probably 1 mole of formic acid is liberated per 7 residues and 1 mole of periodate is consumed per 4 residues. Under similar conditions, formaldehyde corresponding to the presence of 1 reducing end-group per 27 residues has been estimated.

9. Molecular weight by viscosity measurements and Barger's method indicate an apparent M.W. of less than 3,000 i.e. 20 residues.

10. A straight-chain molecule 20 residues in length is proposed for laminarin. This structure explains most of the experimental results with the chief exception of reducing power which indicates a molecular size of some 40 residues. Possible reasons for the discrepancy in reducing power are discussed.

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